CONTROL OF NEURITE OUTGROWTH FROM AVIAN SENSORY GANGLIA

GRAEME J. BRYCE

Ph.D. University of Edinburgh 1992

DECLARATION

I declare that the work described in this thesis and its composition are entirely my own.

ACKNOWLEDGEMENTS

I thank my supervisor Dr. Richard Ribchester for his support and encouragement throughout my studies and for the freedom and flexibilty he has allowed me in my work. Thanks also to Brendan McGrory for printing the photographs.

Thanks to Tony for his humour and understanding, and to Craig whose support over the last three years has enabled the work to be done. And finally I would like to thank my family.

ABSTRACT

Methods used for studying neurite outgrowth in culture range from attachment of dissociated neurons or explants to coated substrata, to embedding cells or tissues in three-dimensional matrices. Here I describe a new technique in which isolated chick embryo dorsal root ganglia (DRG) can be cultured floating on the surface of serum-free culture medium.The utility of the preparation for studies of neurite growth at the cellular level, and at the level of pattern formation during development is demonstrated.

The quantitative effects of neurotrophic factors in regulating neurite outgrowth can be measured using this new preparation. The extent of outgrowth from floating DRG was compared with that from ganglia adhered to laminin, or to polylysine. This preparation provides a substrate-independent assay for neurotrophic factors which offers a number of advantages over existing bioassays. It is also potentially useful in the search for novel neurotrophic molecules.

The movement of neuronal growth cones is not well understood. Cytoskeletal dynamics and cell-substratum adhesion are involved. Since the growth of neurites from floating ganglia occurs in the absence of a conventional substratum it was thought that this preparation might provide new insight into the mechanism of growth cone movement and neurite elongation. Experiments were carried out using the drugs colcemid and nocodazole which affect microtubules and cytochalasin B which affects actin filaments. Colcemid and nocodazole inhibited neurite outgrowth from both floating and adhered ganglia. In contrast cytochalasin B did not inhibit

outgrowth from floating ganglia although it completely inhibited outgrowth from ganglia adhered to laminin.

During the development of sensory innervation, segmental differences arise in the rates of growth of axons invading limb and non-limb regions. Growth rates of axons invading developing limb are faster. It is not known how these different axonal growth rates are controlled. Isolated DRG from different segmental levels were cultured at the air-medium interface. This technique removes potential substrate derived growth regulating factors. No evidence was found for intrinsic determination of different axonal growth rates, although segmental differences were seen in ganglia isolated from older embryos.

The influence of peripheral target tissues in controling outgrowth from DRG was investigated by culturing ganglia and target tissue explants in close proximity. Results suggest that the earliest neurite outgrowth from chick embryo DRG is stimulated by a soluble factor produced by embryonic ectoderm.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Nerve cells are very precisely connected to each other and to different targets in the periphery. It is essential that the correct connections are made during development in order for the adult nervous system to function properly. During development neurons extend processes called neurites which grow towards the cells target where they make permanent connections with specific cells. If adult peripheral nerves are cut, processes from mature neurons can grow back to reform specific synapses. The growth of neurites is therefore of fundamental importance when considering development and regeneration in the nervous system. It is an important challenge of developmental neurobiology to understand how developing neurites find and form synapses with appropriate targets.

The vertebrate nervous system is composed of many neurons and glia of a wide variety of types. The organization of these cells into a functional nervous system requires their assembly in a definite and exact relationship with one another and with the other tissues of the body. As the embryo develops, specific cell types differentiate at different locations and must often form connections over considerable distances. Historically, neurobiologists have been interested in the controls for directing nerve growth: whether they reside in the extracellular environment through which the neurite grows, and if so what is the nature of the signals, or are the expression of an intrinsic neuronal property. The following sections

consider: the development of segmental sensory innervation, from dorsal root ganglion formation to the establishment of sensory innervation patterns and the types of guidance cues which may operate; and the cellular and cytoskeletal basis of growth cone motility and axonal elongation.

THE DEVELOPMENT OF SEGMENTAL SENSORY INNERVATION

The avian limb has proved to be a useful system for studying the control of neuronal growth during development. Beginning with the classic embryological studies of Hamburger and Levi-Montalcini (for example Hamburger 1934, 1939; Hamburger & Levi-Montalcini 1949), much information has been obtained about the development of both sensory and motor innervation. How populations of neurons make divergent pathway choices and the nature of the guidance cues involved is the focus of contemporary study.

The development of dorsal root ganglia

The sensory innervation of the periphery is supplied by axons projecting from dorsal root ganglia (DRG). These originate from the neural crest, a transient structure on the dorsal neural tube that also gives rise to other neuronal populations such as autonomic ganglia (Le Douarin 1982). Neural crest cells migrate along laminin pathways and invade the sclerotome on the ventro-medial aspect of the somites, where they condense to form DRG (Bronner-Fraser & Lallier 1988; Rickmann *et al* 1985). The ganglia are paired, segmentally arranged structures located between cervical and sacral segmental levels. The segmental pattern is

imposed by the somites, the anterior halfs of which contain glycoproteins which inhibit neural crest cell migration, DRG formation and sensory neurite outgrowth (Tosney 1988; Stern & Keynes 1987; Davies *et al* 1990).

Sensory neurons in the DRG innervate two main categories of target: skin, innervated by cutaneous sensory receptors; and muscle, innervated by a number of types of sensory fibres. Morphologically, two populations of cells can be seen in early embryos: the large, fast growing cells in the ventrolateral region of the ganglion (VL cells) and the smaller dorsomedial cells (DM). It was suggested that these two cell populations corresponded to the two different types of target (Visintini & Levi-Montalcini 1939). However, Honig (1982) using horse-radish peroxidase (HRP) to retrogradely label cells in DRG showed that populations innervating skin and muscle were not segregated within the ganglion.

Condensation of neural crest cells to form the DRG anlage occurs during the first 2.5 days of embryonic development in the chick (Weston 1963). This is followed by a period of neuronal differentiation and proliferation accompanied by cell death and outgrowth of processes. Ventrolateral cells differentiate early: between E 2.5-8 (days 2.5-8 of incubation); whereas dorsomedial cells differentiate later: between E 9-15 (Hamburger & Levi-Montalcini 1949). Differentiation is accompanied by proliferation, with mitotic activity in the ganglia reaching a peak on E 5-6 (Levi-Montalcini & Levi 1943; Carr & Simpson 1978a). Cell death in developing DRG begins while proliferation and differentiation are still going on. Naturally-occurring neuronal death is a common phenomenon during development of many neural populations and is thought to ensure that neuron number is matched to target field size (Purves 1988). Hamburger & Levi-Montalcini

counted degenerating cells in staged embryos. They concluded that cell death occurred only in non-limb ganglia, was almost exclusive to the VL population, and was virtually completed by E 7. A more detailed autoradiographic investigation by Carr & Simpson (1978a) showed that neuronal death was a feature of VL and DM cell populations in both limb and non-limb ganglia. Degeneration began at E 4.5 and rose continually in DM cells until E 9.5 (the furthest stage used in their study), but peaked at E 5.5 in VL cells. From these results it is clear that cell death, proliferation and differentiation occur simultaneously in DRG. In contrast, during development of motoneurons proliferation and degeneration are separated by 2-3 days (Hollyday 1983).

Axonal outgrowth

Once differentiated, a primitive neuroblast will begin to extend neurites. As early as E 3 cellular processes were observed on ventrolateral DRG neurons by Ramon y Cajal (1929), although at this stage their growth cones had not yet left the ganglion. Primitive neuroblasts have a bipolar morphology: one process projecting towards the dorsal horn of the neural tube, the other towards the periphery. By E 3.5 peripherally directed processes have entered the proximal environment of the limb (Bennet *et al* 1980; Hollyday 1983; Tosney & Landmesser 1985), where they appear to " wait " until further growth cones join them and a plexus forms. Landmesser (1987) proposed that axons do not enter the limb at this stage due to the growth inhibiting properties of early limb bud mesenchyme. Oakley & Tosney (1991) have shown that proximal limb mesenchyme binds the lectin peanut agglutinin at this early stage. This lectin is known to label cell surface glycoproteins which inhibit sensory

neurite growth *in vitro* (Davies et al 1990). The consequent " waiting " period permits formation of a plexus of axons in which considerable sorting out occurs (Scott 1987). By E 4.5 peanut lectin no longer binds to limb bud mesenchyme (Oakley & Tosney 1991) and sensory axons begin to leave the plexus and invade the limb bud. Sensory axons grow out together with motor axons and mixed nerve trunks form (Landmesser & Honig 1986; Tosney & Landmesser 1985; Swanson & Lewis 1982). By E 5.5 the dorsal and ventral nerve trunks are present in the developing wing (Swanson & Lewis 1982) and the four major cutaneous nerve branches have formed by E 7 (Martin *et al* 1989). Visintini & Levi-Montalcini (1939) demonstrated that spinal motor reflexes can be elicited by cutaneous stimulation as early as E 6, thus indicating that some sensory axons have formed connections with skin at this stage and that the centrally directed processes of these cells have entered the spinal cord. Martin *et al* (1989) showed that there is little change in the basic innervation pattern after E 9, although further arborization of cutaneous nerve endings persists for some time.

Much less is known about the development of central projections of DRG sensory neurons. Smith (1983) used HRP labelling techniques to show that the central projections of thoracic sensory neurons in the bullfrog are accurate from the outset. The development of central projections of lumbosacral sensory neurons was studied in the chick by Davis *et al* (1989) again using HRP. They showed that afferent fibres reach the spinal cord by E 4, and project in the primordium of the dorsal funiculus both rostral and caudal to their point of entry during E 4.5-5. They then appear to " wait " for 24 hr before invading the gray matter at E 6. The reason for this pause is unclear but may depend on the peripheral connections made

by DRG neurons which occurs at E 5.5-6 (Scott 1982). Central connections of sensory neurons are regulated by their peripheral contacts in the frog (Smith & Frank 1987) and it may be that contact with peripheral targets is required to form appropriate central connections. Davis *et al* (1989) found that functional contacts form between sensory neurons and motoneuron dendrites at E 7.5, although this is somewhat later than Visintini & Levi-montalcini (1939) suggest.

Sensory neurons in DRG undergo a distinct morphological change at around E 3.5-4.5, prior to sending axons into the limb (Panesse 1972). This change also occurs in culture: small, spindle-shaped, phase-dark neuronal cell bodies become phase-bright and spherical as they extend neurites. Recent evidence suggests this morphological change is controlled by neurotrophic factors (Wright *et al* 1992).

The experiments which will be described later were carried out at E 4.5, 5.5, 6.5 and 8. At these developmental stages ganglia contain varying numbers of proliferating and degenerating neurons. In addition, at E 5.5-8 ganglia also contain neurons innervating central and peripheral targets.

Sensory innervation patterns

An outline of the origin and development of dorsal root ganglia and the time course of innervation has been given. I will now briefly discuss the patterning of skin sensory innervation.

Electrophysiology and retrograde HRP tracing techniques provide evidence that chick sensory neurons grow from the outset to their target dermatomes with few errors (Scott 1982, 1987). A dermatome is the region of skin innervated by a single DRG. On the trunk, dermatomes show a simple segmented innervation pattern. The situation on a limb is more complex however. Scott (1982) studied the development of limb dermatomes. She showed that axons from several DRG mix and cross over in the plexus which forms in the proximal region of the limb bud prior to axonal invasion. Each cutaneous nerve which leaves the plexus contains axons from several adjacent DRG. Thus cutaneous axons from a single DRG project to the periphery along several different nerves. When they reach the skin the fields of individual cutaneous nerves merge to form the dermatome. Since each nerve contains axons from several DRG, the dermatomes of adjacent DRG overlap. The location of dermatomes and the composition of cutaneous nerves were found to be almost identical from early developmental stages (prior to the peak period of cell death) through to maturity (Scott 1982; Honig 1982). It appears from these results that skin sensory axons grow directly and precisely to the correct targets during development.

Axonal guidance and pathway selection

In the developing embryo, growth cones are able to navigate accurately over considerable distances in order to form functional connections. The study of this problem and the revelation of the cues involved is one of the most important areas of developmental neurobiology. There is evidence to support several mechanisms of axonal guidance. Experiments which show that the morphology and rate of growth of neurons *in vivo* and *in*

vitro is similar suggest that some aspects of neuronal form are intrinsically determined. Other evidence suggests that diffusible molecules such as growth factors may guide developing neurites. Patterns of differential adhesivity have also been demonstrated to affect neuronal elongation. Extracellular matrix molecules and cell surface glycoproteins which mediate cell-substrate adhesion have been identified and demonstrated to regulate axonal pathfinding. Some extracellular matrix and cell surface molecules inhibit growth cone advance, thus the extracellular substrate may present the growing axon with opposing types of cues. Galvanotropism of neurites and growth cones has been demonstrated *in vitro*. Contact guidance in which cells detect and respond to topographical features may also have a role. Some of the evidence supporting each of these hypotheses is discussed in the sections below.

Intrinsic determination of neuronal form or environmental regulation

There is evidence that some aspects of axonal growth are genetically predetermined. The morphology of single isolated neurons growing *in vitro* is often similar to that of neurons *in vivo* (Cowan & Banker 1979; Acklin & Nicholls 1990). The simplicity of the extracellular environment *in vitro*, and in particular the absence of specific guidance cues, suggests that the observed similarity results from an intrinsic determination of certain components of neuronal shape. The morphologies of neurons growing *in vitro* and *in vivo* are not identical, similarities generally only existing in the branching patterns of the major neurites. The multiplicity of extrinsic factors present *in vivo* control the final shape of the neuron, and ensure the specificity of formation of connections.

There is evidence that the growth rates of cranial sensory neurons may be intrinsically determined. Davies (1989a) cultured isolated neurons from chick embryo cranial sensory ganglia, and showed that their growth rates *in vitro* were similar to those observed *in vivo*. He also showed that the rate of growth differed according to target distance *in vivo* and that this difference was preserved *in vitro*. During the development of sensory innervation, axons invading developing limbs must elongate over greater distances than those which innervate non-limb regions. Consequently segmental differences arise in the rates of neurite outgrowth from DRG located at different axial levels, with axons innervating developing limb buds growing faster than those innervating non-limb regions (see Swanson & Lewis 1982). It is not known how these differential growth rates are controlled: intrinsic determination, as in the case of cranial sensory ganglia; or environmental regulation.

Experiments in which the normal spatial relationship between DRG and the limb was altered by microsurgery *in ovo* prior to sensory neuron outgrowth suggest that guidance of sensory axons is determined by environmental signals rather than by intrinsic properties of the neurons. Hamburger (1939) grafted ectopic limbs onto host embryos at early developmental stages and found that foreign nerves were attracted into them. He did not distinguish between sensory and motor nerves. The pattern of innervation of ectopic limbs was often near normal, leading Hamburger to propose that innervation was controlled by the environment of the limb. Swanson & Lewis (1982) did similar experiments in which limb buds were grafted onto the third brachial arch and a near normal pattern of innervation was observed.

Additional perturbations have been carried out. Honig *et al* (1986) showed that sensory axons could project to their correct targets following " short " rotations of the neural tube. Scott (1986) demonstrated that if only the dorsal neural tube was rotated, sensory innervation patterns corresponded to the new position of the DRG, whereas if the entire neural tube was shifted axon trajectories were appropriate to their original position. Rotation of the dorsal neural tube alone will not affect the projection of motoneurons and this lead Scott to suggest that sensory axons may be guided by motor axons.

Landmesser & Honig (1986) and Scott (1988) found that following early motoneuron ablation the projection of sensory axons was greatly affected: muscle nerves either did not form at all or were much reduced in diameter. This suggests that guidance cues for muscle innervation (by sensory axons) are not located on muscle, but are provided by developing motoneurons. Following motoneuron ablation many of the sensory axons which would otherwise have innervated muscle were found to project instead to skin, suggesting that the trajectories of individual sensory axons are not pre-specified. Martin *et al* (1989) ablated regions of ectoderm and found that mixed nerve trunks and muscle nerve branches developed as normal but cutaneous nerve branches failed to form. Thus specific signals may be present in developing skin which attract cutaneous nerves.

It is likely that a multiplicity of spatially and temporally expressed guidance cues operate within the limb. The possibility that both intrinsic and environmental factors influence the rate of outgrowth of axons from DRG is one of the issues addressed experimentally in this thesis.

Diffusible factors

Chemical attractants diffusing from targets and guiding the growth cones of developing axons would appear to provide an elegant solution to the problems of axon guidance. Many factors have been identified which support the growth and survival of developing neurons and promote the outgrowth of their neurites, although the capacity of such factors to act as specific chemoattractants, guiding growth cone navigation from a distance, remains unproven. Nerve growth factor (NGF) is required for the survival of developing peripheral sympathetic and sensory neurons: if endogenous NGF is neutralized by specific anti-NGF antibodies then these neurons die (Levi-Montalcini & Booker 1960; Levi-Montalcini 1964). NGF stimulates outgrowth from isolated sympathetic and sensory ganglia and dissociated cells in culture, and many workers have suggested that NGF acts as an attractant and may have a specific neurotropic role during development. Dorsal root ganglion explants in culture show more extensive outgrowth from the side facing a source of NGF (Charlwood *et al* 1972; Ebendal & Jacobson 1977), and Campenot (1982) has demonstrated that NGF can selectively maintain sympathetic neurite branches in its locale. Sensory neurites will orient within an NGF gradient towards higher concentrations (Letourneau 1978a), and Gunderson & Barret (1979, 1980) showed that growth cones of DRG neurons *in vitro* respond to a point source of NGF by turning and growing towards it. Further evidence that NGF may attract neurons to peripheral targets comes from Menesini-Chen *et al* (1978) who injected NGF into the brainstem of neonatal rats and found abnormal growth of sympathetic axons into the spinal cord and along the dorsal columns to the injection site.

However, in many of these experiments extremely high concentrations of NGF were used. For example the turning response of DRG growth cones observed by Gunderson & Barret was towards an NGF concentration >25 000 times the maximum reached in the most densely innervated cutaneous target field of the mouse embryo (Davies *et al* 1987; Davies 1987). At such high concentrations NGF adsorbs onto culture surfaces, increasing adhesivity (Pearce *et al* 1973; Schubert & Whitlock 1977). As growing neurites are known to prefer more adhesive substrata (Letourneau 1975b) the turning response of neurites to NGF may be the result of increased substrate adhesivity rather than specific attraction. Campenot (1982) cultured sympathetic neurons using a compartmentalized chamber in which the extracellular environments around the cell body and growing neurites were separated. His results demonstrated that NGF can selectively maintain the neurites in its locale. In the light of this study Davies (1987) reassessed the work of Charlwood *et al* (1972), Ebendal & Jacobson (1977), Letourneau (1978a), and Menesini-Chen *et al* (1978). If outgrowth of neurites or axons from ganglia or explants was initially random, and only those which grew in the direction of an NGF source were maintained, this would result in more outgrowth being seen from the side facing the NGF source. Thus Davies suggested this result may be due to selective maintainance of neurites rather than evidence for specific attraction.

Nerve growth factor is an unlikely molecule for neuronal chemoattraction. NGF lacks both neuron and target specificity, and is synthesized late on in the course of innervation. Davies *et al* (1987) showed that NGF synthesis in the cutaneous target fields of trigeminal ganglia, and NGF receptor expression by neurons does not occur until after nerve fibres have

reached the target field. Wyatt *et al* (1990) used more sensitive probing techniques and found that mRNA for the NGF receptor was expressed by sensory neurons prior to and during innervation. However, it has been shown that survival and growth of sensory neurons is independent of NGF prior to target innervation (Davies & Lumsden 1984; Vogel & Davies 1991).Thus a role for NGF as a chemoattractant during early phases of axonal outgrowth is unlikely, although NGF may have a neurotropic function during the final stages of sensory axon arborization within the target field (Martin *et al* 1989).

NGF is one of a family of molecules, recently named " neurotrophins ", that support the growth and survival of developing neurons (for review see Ebendal 1992). The amino acid sequences for the members of the neurotrophin family are strikingly similar. In particular, six cysteine residues which form three intramolecular bonds, are common to all (Ebendal 1992). The neurotrophins brain derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3), have been shown to support the growth and survival of sensory neurons. BDNF was initially isolated from brain extract (Barde *et al* 1982) and affects sensory neurons derived from the neural crest and ectodermal placodes both *in vivo* and *in vitro* (Lindsay *et al* 1985; Davies *et al* 1986; Barde *et al* 1987; Kalcheim *et al* 1987). Yip & Johnson (1985) found that severing the dorsal roots of newborn rats caused considerable cell death in the affected DRG. They proposed that DRG neurons required a centrally derived survival factor which they suggested might be NGF. Davies *et al* (1986) demonstrated that the centrally derived trophic factor required by sensory neurons (in this case trigeminal ganglia) was BDNF. One population of CNS neurons, retinal ganglion cells, have also been reported to respond to BDNF *in vitro*

(Johnson *et al* 1986). Evidence from the above systems suggests a role for BDNF as a neurotrophic (survival) factor, rather than as an attractant. Current evidence also suggests that the main function of NT-3 is as a survival factor (Hohn *et al* 1990; Maisonpierre *et al* 1990) although Wright *et al* (1992) have recently shown that BDNF and NT-3 also promote the morphological maturation of DRG sensory neurons *in vitro*. Two other members of the neurotrophin family have been isolated using genetic cloning techniques: NT-4 (Hallbook *et al* 1991) and NT-5 (Berkemeier *et al* 1991). As yet, little is known of their function.

There are many candidates for neuronal chemoattractants among other molecules recently discovered to possess neurotrophic activity. These include neuroleukin, epidermal growth factor, fibroblast growth factor, and insulin-like growth factor (Gurney *et al* 1986; Morrison *et al* 1987; Walicke *et al* 1986; Recio-Pinto *et al* 1986). As yet there is no compelling evidence that these molecules are chemoattractive for neurons.

There is evidence, however, for chemoattractant activities during neuronal development. The crucial demonstration comes from co-culture experiments in which un-innervated target tissue can induce and orient primary neurite outgrowth from specific neurons. The experiments are carried out in three-dimensional collagen gel matrices, which not only provide a permissive substratum for neurite growth, but will also stabilize diffusion gradients. Lumsden & Davies (1983, 1986) co-cultured mouse trigeminal ganglia and its peripheral target field (the epidermis of the maxillary process). Neurite outgrowth was profuse and was directed exclusively towards the target explant. Substituting maxillary process with hyoid process or limb epidermis did not elicit outgrowth. These results

suggest that the maxillary process produces a diffusible factor which directs specific axon outgrowth toward it. Commisural axons in the spinal cord project directly to the ventral floor plate during development. Tessier-Lavigne *et al* (1988) co-cultured floor plate explants with explants of dorsal spinal cord in 3-D collagen matrices and found that commisural neuron outgrowth was attracted to the floor plate. In neither of these systems has the diffusible molecule been identified.

In the chick limb bud sensory axons are able to project directly to appropriate targets even after the neural tube has been rotated or parts of the limb selectively ablated (Honig 1986; Scott 1986; Landmesser 1984). This suggests that specific chemoattractants may be involved. There is evidence that limb bud tissue can stimulate neurite outgrowth *in vitro*. Pollack and colleagues, working with amphibians, showed that spinal cord neurite outgrowth was stimulated by limb bud mesenchyme in co-culture (Pollack & Leibig 1977; Pollack & Muhlach 1981). They also found that outgrowth from DRG was stimulated by spinal cord explants and limb bud mesenchyme (Pollack *et al* 1979, 1980). Nurcombe & Bennett (1983) showed that spinal cord neurite outgrowth was also stimulated by limb bud mesenchyme in the chick from stage 24 to 36. They did not study outgrowth from DRG. Ebendal & Jacobson (1977) cultured ganglia from E 10 chick embryos with explants of a wide variety of tissues. They observed that growth from sympathetic ganglia was stimulated by many tissue explants including spinal cord, skin, and colon. DRG in their cultures showed dense outgrowth of fibroblast-like cells and were not included in quantitative analysis. They do however state that outgrowth from DRG appeared to be stimulated by heart explants (and presumably, therefore, not by any other explants, although this is unclear from their study).

Most of the explant co-culture studies described above were conducted late in development using regenerating neurons and denervated target tissues. Consequently little information was gained about developmental processes. In addition, none of the above experiments were carried out in 3-D collagen matrices so it is not possible to conclude that specific attractant molecules are present in limb tissues, only that neurite promoting or cell survival factors appear to exist.

The mechanism of action of neurotrophic factors has been studied almost exclusively using NGF. Hendry *et al* (1974) and Stoekel *et al* (1976) showed that NGF is taken up by nerve endings and transported to the cell body where it influences RNA and protein synthesis (Burstein & Greene 1978). The regulation of growth cone formation and function by NGF has been studied primarily in PC12 cells. Application of NGF up-regulates the expression of a number of molecules involved in regulating growth cone motility and neurite elongation: GAP-43 (Costello *et al* 1990), the cell adhesion molecule L1 (Salton *et al* 1983), laminin receptors (Rossini *et al* 1990), and protein kinase C (Van hoof *et al* 1986).

To summarize: target derived neurotrophic factors have an important role in the development and maintainance of specific neuronal populations. There is some debate as to whether known factors such as NGF can also act as chemoattractants and guide growing axons towards their targets. However, there is evidence from the trigeminal system and from the development of commisural axons in the spinal cord which supports the chemoattraction theory of axonal guidance. One hypothesis addressed in the present thesis is that sensory innervation of limb buds may also be controlled by attractant molecules.

Differential adhesion

The role of adhesion to a substratum in axonal elongation was first recognized by Harrison (1907, 1910) in studies of neurites growing *in vitro* through plasma clots or along spiders webs. The neurites always grew along the solid fibrils of the substrate. Further studies revealed that growth cones could contact, adhere to and pull on other cells or objects in their environment (Nakai & Kawasaki 1959; Letourneau 1975a). Yamada *et al* (1970) demonstrated that filopodia were required for neurite growth and proposed that extension of filopodia and subsequent adhesion to the substratum regulated neurite elongation. *In vitro* studies of non-neuronal cell locomotion demonstrated that regional differences in substrate adhesivity could affect the rate and direction of movement. Letourneau applied this approach to neuronal cultures and found that differences in substrate adhesivity could regulate the direction of axonal elongation: axons prefered to grow along substrate pathways of highest adhesivity (Letourneau 1975a, b). From these *in vitro* studies Letourneau proposed that regional differences in adhesivity of the extracellular environment could guide axonal elongation *in vivo*. Work by Bray demonstrated the importance of mechanical tension exerted by the growth cone in neurite growth (Bray 1979), and a mechanism was suggested in which the growth cone samples environmental surfaces via filopodia, growth cone-substratum adhesivity then allows tension development and the neurite moves in the direction with the greatest pull ie. the direction with the strongest adhesion (Bray 1982, 1991). Analysis of the pathfinding behaviour of identified neurons in intact insect embryos suggested that growth cones could advance along routes of high adhesivity and up adhesive gradients (Bentley & Keshishian 1982; Bastiani *et al* 1985). A

role for differential adhesion in axonal pathfinding was demanded by these studies. Molecular characterization of the adhesive contacts involved in axonal navigation has been the focus of much study in recent years.

Extracellular matrix molecules and cell surface glycoproteins

Many cell surface and extracellular matrix glycoproteins involved in adhesive interactions have been identified. Cell surface molecules which mediate adhesion include cell adhesion molecules (CAM's) such as N-CAM and L1, cadherins such as N-cadherin, and integrins. Components of the extracellular matrix such as laminin, fibronectin, collagen, and proteoglycans have also been identified.

The role of cell adhesion molecules in neural development has been reviewed by Edelman (1984) and by Rutishauser & Jessel (1988). The first neural cell adhesion molecule to be identified was NCAM (Thiery *et al* 1977). This glycoprotein comprises a single polypeptide chain, highly variable in both its protein and carbohydrate structure. Variations in glycosylation typically involve sialic acid content and the adhesive properties of NCAM are thought to be modulated by polysialic acid. NCAM mediates cell-cell adhesion via homophilic interactions between the surfaces of the cells involved. It is expressed on neurons, glial cells and muscle with different forms of the molecule present at different developmental stages. NCAM binding, unlike some other forms of cell adhesion, is not calcium (Ca) dependent. One of the functions of NCAM in development is as a guidance cue during axonal projection. For example, when axons of chick retinal ganglion cells leave the eye they follow a stereotyped route to the tectum (Silver & Sapiro 1981). During this journey

growth cones are seen in close apposition to the end feet of radial epithelial cells; in addition axons from one region of the retina remain close to each other as they grow. NCAM has been found to be preferentially expressed on the end feet of radial glia and addition of anti-NCAM *in vivo* results in disrupted axonal projection. Anti-NCAM also causes a reduction in axon-axon adhesion (Silver & Rutishauser 1984).

Another cell adhesion molecule implicated in axon guidance is L1 (also known as NgCAM). This glycoprotein is expressed predominantly on axons and is thought to have a major role in fasciculation (Grumet & Edelman 1984). Antibodies against L1 promote defasciculation *in vitro* and inhibit axonal elongation along pre-existing axon fascicles (Chang *et al* 1987).

Besides Ca-independent cell adhesion molecules such as NCAM and L1 there is also a class of Ca-dependent cell adhesion molecules given the general name of cadherins. They are termed Ca-dependent as they are resistent to proteolysis in the prescence of Ca^{2+} ions, but susceptible to degradation when Ca is removed. These molecules will only function as cell adhesion molecules if Ca is present (Takeichi 1987). The neuronal cadherin, N-cadherin mediates cell-cell adhesion via heterotypic binding to specific receptors. N-cadherin is thought to be involved in neurite outgrowth during development. Antibodies which inhibit N-cadherin-mediated adhesion have been shown to prevent outgrowth of neurites on cultured astrocytes (Tomaseli *et al* 1988).

Cell adhesion molecules are diferentially expressed during axonal growth into developing limb buds. NCAM expression is low on sensory axons and

high on motor axons (Landmesser 1991), whereas N-cadherin is high on cutaneous afferents and low on motoneurons and muscle afferents (Hatta *et al* 1987). Thus some degree of axonal sorting may be achieved in this way, but this is unlikely to explain how individual growth cones select divergent pathways.

Integrins are a family of cell surface glycoproteins, composed of α/β heterodimers, that mediate cell-substratum adhesion by binding to extracellular matrix molecules such as fibronectin, laminin and collagen (Bozyczko & Horwitz 1986). Many integrins are non-specific and will bind to any extracellular matrix (ECM) molecule containing the appropriate cell binding domain. For a growing neurite to be able to respond to guidance cues in the ECM it must express integrins.

The role of extracellular matrix molecules in neuronal development has been reviewed by Sanes (1989). A growing axon must travel through an environment containing matrix molecules, which may be secreted or bound to cell surfaces. Many matrix molecules have been demonstrated to affect axonal growth and pathfinding. In particular, laminin, a major component of basement membrane has a profound effect on neuronal growth (Rogers *et al* 1983; Manthorpe *et al* 1983; Edgar *et al* 1984). *In vitro* studies have shown that neurite outgrowth on a purified laminin substrate is considerably greater than on other ECM molecules such as fibronectin, collagen, and proteoglycans (Carbonetto *et al* 1983; Gundersen 1987). In addition laminin stimulates outgrowth from some central neurons which do not respond to fibronectin or collagen (Rogers *et al* 1983), and may even permit the short term survival of NGF-dependent cells in the absence of NGF (Baron-Van Evercooren *et al* 1982; Edgar *et*

al 1984). It has been demonstrated that pathways of substratum adsorbed laminin can guide growing axons *in vitro* (Hammarback *et al* 1985, 1988; Gundersen 1987). Immunohistochemical studies *in vivo* have shown that laminin is present in several pathways followed by growing axons. For example, along the paths taken by DRG axons entering proximal limb buds (Rogers *et al* 1986) and along the trajectories of primary motoneurons in zebrafish embryos (Frost & Westerfield 1986). The way in which laminin stimulates neurite elongation is not well understood. As well as providing a substrate favourable for adhesion and locomotion it is also thought to stimulate outgrowth directly via a specific receptor mediated interaction (Edgar *et al* 1984, 1988). Bixby (1989) has shown that laminin binding to its receptor activates protein kinase C in cultured ciliary ganglion cells and suggests that the resulting biochemical changes stimulate neurite production.

Besides laminin, some other ECM components promote axon outgrowth. Fibronectin is an effective substrate for sensory and sympathetic neurite outgrowth (Rogers *et al* 1983), and for retinal neurite outgrowth (Akers *et al* 1981). There is evidence that pathways of fibronectin can guide axonal growth *in vitro*, and Yip & Yip (1990) showed fibronectin to be present around nerve roots and in developing chick limbs. Collagen type I and type IV are also effective substrates for sensory neurite growth (Carbonetto *et al* 1983). Proteoglycans and glycosaminoglycans have generally not been found to stimulate axonal growth, although Hantaz-Ambroise *et al* (1987) showed that a substrate coated with heparan sulphate proteoglycan will induce neurite elongation.

Proteoglycans &
Gag's - roles
in conjuaction
with other cues
?

Growth inhibitors

As well as containing molecules which promote neurite outgrowth extracellular substrates are also endowed with molecules that repulse advancing growth cones and inhibit neurite elongation. During the past few years some of these molecules have been identified. For example, Caroni & Schwab (1988a,b; Schwab & Caroni 1988; Schwab 1990) have isolated two glycoproteins of 35 kD and 250 kD from oligodendrocyte membranes which cause growth cone collapse and inhibit neurite elongation *in vitro*. Antibodies against these proteins promote axonal regeneration *in vivo* following CNS lesion (Schnell & Schwab 1990). Membrane glycoproteins which bind peanut lectin and inhibit growth cone advance have also been identified in developing somites and these glycoproteins may be responsible for the segmental pattern of sensory and motor innervation (Davies *et al* 1990). Some extracellular matrix components inhibit neurite elongation. Carbonetto *et al* (1983) demonstrated that substrates containing the glycosaminoglycans heparin, chondroitin sulphate or hyaluronic acid were unable to support neurite growth. Verna *et al* (1989) showed that growing neurites would avoid patches of culture substrate coated with chondroitin sulphate and that developing sensory nerves were unable to penetrate the epidermis of developing skin due to the presence of a barrier of chondroitin sulphate (Verna 1985; Fischard *et al* 1991). Oakley & Tosney (1991) found that chondroitin sulphate and glycoproteins that bind peanut lectin are present in developing chick limbs in regions avoided by growing axons. Disappearence of these molecules allowed axons to invade these regions. Fibronectin, which stimulates neurite outgrowth in some systems, has been found to inhibit growth of primary motoneurons in zebrafish embryos (Frost & Westerfield 1986).

Thus, the extracellular substrate may present a growing axon with many opposing types of cues. This may permit differential guidance: different growth cones responding to different cues. The existence of multiple extrinsic guidance molecules suggests that the environmental influences on a growth cone may not be constant but may change spatially and temporally during development. Most of these issues are not addressed further in the thesis, although some data on the role of laminin and cell-surface interactions is presented.

Galvanotropism

Galvanotaxis is a directed cellular response to an electric field. Galvanotaxis of neural crest cells and myoblasts and galvanotropism of neuronal growth cones have been demonstrated *in vitro* (Hinkle *et al* 1981; Patel & Poo 1982; McCaig 1986, 1989). These effects are believed to occur at physiological electric field strengths (Patel *et al* 1985). The cellular mechanism mediating this response is not clear. An applied electric field induces asymmetries of integral membrane proteins (Poo & Robinson 1977) and cytoskeletal components (McCaig & Dover 1989) which may result in asymmetries of cell-substrate adhesion and tension development thereby directing growth cone advance. The role of electric fields in directing neurite outgrowth is not considered further in the present thesis.

Contact guidance

Growing axons are thought to be sensitive to topographical features in their environment. Ebendal (1977) observed that axons growing out from

dorsal roots *in vivo* were associated with and appeared to be oriented by fibrillar extracellular matrix material. He prepared substrates coated with aligned collagen fibrils and observed that growing neurites became oriented by such substrates *in vitro* (Ebendal 1976). Chemical, mechanical and topographical cues are provided by such surfaces and it is unclear which of these properties determines neurite behaviour. Clark *et al* (1987) used microfabrication techniques to prepare multiply grooved quartz substrates and observed patterns of neurite outgrowth to be markedly aligned to groove direction provided the grooves were at least 2 μm deep, thus demonstrating that topographic features can guide growing neurites. However, 2 μm grooves are considerably larger in size to those found in fibrillar extracellular matrix (individual collagen fibres have diameters in the range 20-100 nm, though they can form larger aggregates; Dunn & Ebendal 1978). Clark *et al* (1991) used ion etching to prepare quartz substrata with 130 nm grooves. These surfaces mimic the topography of fibrillar ECM and are devoid of chemical and mechanical cues. They found that neurite outgrowth from chick embryo cerebral neurons was unaffected by such topography. This suggests that the topographic component of fibrillar ECM is not important in the guidance of growing neurites. The *in vitro* and *in vivo* observations of Ebendal may therefore be due to the chemical composition of the collagen fibres. Collagen is rich in negatively charged proline residues which may affect cell adhesion (Curtis 1973) and this could result in the oriented patterns of neurite growth.

Summary

The environment through which a growth cone advances contains a broad range of both soluble and bound molecules which can guide axonal

behaviour. Galvanotactic and topographic guidance cues may also be present. It is likely that growth cones will encounter simultaneous influences, some of which may be in opposition. These environmental signals are integrated by the growth cone which can then make a directed response.

The molecular basis of growth cone motility and axonal elongation, which underlies pathfinding capabilities, will be discussed in the next section.

GROWTH CONE MOTILITY AND NEURITE ELONGATION

The enlarged terminal ending of an axon or dendrite is known as the growth cone. This irregularly shaped structure was first seen by Ramon y Cajal over a century ago in silver impregnated histological sections of chick embryos (Ramon y Cajal 1890). Though working with fixed tissue he argued that the growth cone is a highly motile structure. This was confirmed by Harrison (1907) who observed the behaviour of living growth cones in tissue culture and Speidel (1933) who studied the growth cones of living nerves *in situ*. The growth cone extends and retracts actin-containing surface extensions known as filopodia (or microspikes) and lamellipodia (or veils). These have a similar ultrastructure and content to those at the leading edge of other migrating cells such as neutrophils and fibroblasts (Singer & Kupfer 1986). The abilities of the growth cone to crawl, to explore, to exert tension and to follow guidance cues enables developing neurites to reach their proper targets. These abilities depend on the actin based activity of filopodia and lamellipodia and accompanying cytoskeletal remodelling (Lockerbie 1987; Mitchison & Kirschner 1988; Smith 1988; Bray 1991).

The neuronal cytoskeleton

There are three major classes of cytoskeletal components in neurons: actin microfilaments, microtubules and the neuronal class of intermediate filaments the neurofilaments. Microtubules and neurofilaments are the major structural components of the axon. Neurofilaments (8 to 14 nm in diameter; composed of a triplet of different subunits of approximately 68, 160 and 200 kD; Lazarides 1980) rarely project into the growth cone. Microtubules (22 to 25 nm in diameter; hollow cored tubular polymers of a heterodimeric subunit composed of α and β tubulin; Kirschner & Mitchison 1986) extend through the central region of the growth cone and end close to the actin rich domain around the leading edge. Microtubules in axons exist in short lengths. Individual microtubules in cultured sensory neurons are about 100 μm long (Bunge & Bray 1981), in mature peripheral nerves they may be up to 500 μm long (Tsukita & Ishikawa 1981). Axonal microtubules are arranged with their fast growing ends ("plus" (+) ends) farthest from the cell body (Heidemann *et al* 1981; Mitchison & Kirschner 1988). Actin filaments (7 nm in diameter; helical polymers of G-actin, a 42 kD globular protein; Pollard & Cooper 1986) form a meshwork within the distal regions of the growth cone, and bundling of parallel filaments occurs to form the core of filopodia. Filamentous actin also forms the cortical cytoskeleton, an intricate network of actin and associated proteins attached to the inner face of the plasma membrane (Smith 1988). Each type of cytoskeletal filament is associated with a spectrum of specific accessory proteins that regulate the functioning and assembly of the cytoskeleton and are themselves regulated by Ca^{2+} and second messenger systems. Many cytoskeletal elements are linked to the plasma membrane, and via integral membrane proteins to the extracellular

environment. This relationship is such that changes in the molecular or ionic composition of the external milieu of the growth cone is able to affect assembly and organization of cytoskeletal components within the cell. Consequently the crawling, explorative, tensile and pathfinding behaviour of the growth cone is ultimately directed by the environment through which it grows (Bray & Hollenbeck 1988; Kater *et al* 1988; Smith 1988).

Growth cone motility

The motility of the growth cone and the process of axonal elongation are distinct processes whose integration results in growth and target innervation by developing neurites. What controls the protrusion and motility of filopodia and lamellipodia on the growth cone? Yamada *et al* (1970) found that cytochalasin B inhibits the formation of these cellular processes and abolishes protrusive activity at the growth cone. The primary effect of this drug is to attach to the barbed ends of actin filaments and so prevent actin polymerization (MacLean-Fletcher & Pollard 1980). Binding can also lead to the severing of actin filaments (Hartwig & Stossel 1979). A role for actin in generating filopodia and lamellipodia is strongly implied by these findings. Filopodial extension could result simply from actin polymerization (Tilney & Inoue 1982) or from a more complex process of reversible gelation and solation of three-dimensional actin gels (Oster 1988; Oster & Perelson 1987). Tilney & Inoue (1982) suggested that actin subunits simply add to the forward end of an actin filament adjacent to the cell surface, thus pushing on the membrane. Oster (1988) proposed that cross-linking of actin filaments to form a gel and subsequent solation could result in leading edge protrusion. The possibility that osmotic pressure could produce the driving force for filopodial growth was

tested by Bray *et al* (1991). They examined the response of neurons growing in tissue culture to changes in osmolality of the surrounding medium. They found that reductions in osmolality of the medium sufficient to more than double the internal osmotic pressure had no effect on the formation of filopodia and lamellipodia. It therefore seems unlikely that an internal hydrostatic pressure is responsible for protrusive events at the growth cone. Smith (1988) and Bray (1991) in their reviews of the cytoskeletal basis of neurite growth prefer Oster's gel-sol model to explain filopodial formation on the basis of current evidence.

Observations of growth cones in culture marked with small particles of glass has revealed patterns of movement of the surface (Bray & Chapman 1985). Small particles are carried rearward at rates greater than the forward advance of the neurite as a whole. This retrograde movement on the dorsal surface of the growth cone is thought to be due to a steady movement of the cortical actin network associated with the plasma membrane. Observations of many cell types *in vitro* have shown this to be a general feature of the locomotion of cells over surfaces (Singer & Kupfer 1986). How these steady movements of actin are generated is not clear but is thought to result from a myosin-generated contraction at the base of the growth cone. Letourneau (1981) demonstrated that myosin-II is present in the growth cone. However, there is no direct evidence for myosin-II involvement in generating the necessary force for cortical actin movement, although potentially it could (Letourneau & Shattuck 1989; Bray 1991). The function of rearward transport of the upper surface of the growth cone is generation of tension which is believed to play a major role in growth cone guidance (Bray 1987, 1991). Rearward flow of membrane across the growth cone must be internalized by endocytosis at the base of

the growth cone. Addition of new membrane in growth cones occurs at the tips of filopodia (Feldman *et al* 1981) and via association with the actin cortex flows rearward to the base of the growth cone. Cheng and Reese (1987) labelled growing neurites with cationized ferritin for various times then quick froze and examined the tissue using electron microscopy. They found that endocytosis occurs at the base of the growth cone and internalized membrane vesicles are then available for recycling to the tips of filopodia.

Axonal elongation

Biosynthesis in a neuron occurs in the nucleus, endoplasmic reticulum and Golgi apparatus, which are located in the cell body. Extension of axons and dendrites requires the directed delivery of membrane, cytoskeletal components and other proteins, which are then assembled into the growing neurite. Most evidence suggests that assembly occurs at the tip. The earliest evidence for tip assembly comes from observing the behaviour of isolated growth cones. If the growth cone of a neurite in culture is severed from the rest of the cell it will continue to elongate for several hours (Hughes 1953; Shaw & Bray 1978). This elongation occurs in the presence of inhibitors of protein synthesis and is thought to utilize precursor materials stored in the growth cone. Further evidence comes from experiments in which neurons growing in culture are labelled with particles. Bray (1970) labelled growing neurites with glass beads. As the neurites grew the beads were left behind, implying that assembly occurs at or near the tip. Since the beads in this experiment label only the plasma membrane this tells us that assembly of membrane occurs at the tip. Studies using H-fucose, which labels membrane glycoproteins, to follow

membrane incorporation in regenerating rat sciatic nerve also show that membrane addition occurs at the growth cone (Griffin *et al* 1981).

Besides plasma membrane, axons contain a scaffold of microtubules and neurofilaments. Are these filaments also assembled at the tip of a growing axon? This question was addressed by Black & Lasek (1980) who oserved that radioactive tubulin synthesized in the cell body was carried into the axon at a steady rate. They suggested that microtubules were assembled in the cell body and pushed out into the developing axon. However, many axons and neurites are highly branched, and Zenker & Hohberg (1973) estimated that the number of microtubules in an entire axonal arbor would be several times the amount which emerge from the cell body. More recent work has provided evidence that microtubule assembly occurs at the tips of growing axons. Bamburg *et al* (1986) applied microtubule depolymerizing drugs locally at the tips of axons growing *in vitro* and found that growth was inhibited. Local application at the cell body did not inhibit growth, demonstrating that microtubule assembly occurs at the tip. Okabe & Hirokawa (1990) analysed the recovery of fluorescence after photobleaching fluorescently labelled microtubules in the axons of cultured neurons. They observed that the bleached zones did not move along the axon as growth occurred demonstrating that axons are not pushed out from the cell body. However they also found that all bleached zones recovered indicating that microtubules are not static polymers in the axon and may behave according to the dynamic instability model of Mitchison & Kirschner (1986) even within the confines of a neurite, although it is also possible that flourescence recovery results from local microtubule assembly along the length of the axon.*

How is growth cone activity linked to neurite elongation ?

A number of models have been proposed to explain how movements of the growth cone are related to axonal elongation. Bray has suggested that development of mechanical tension by the growth cone leads to assembly of an axonal process. By studying the shapes of neurons growing on poorly adhesive substrata he deduced that growth cones in tissue culture are able to develop tension and pull against the axons to which they are attached. Bray also showed that if a growing neurite is pulled to one side using a microelectrode then the direction of growth changes in line with the new tension (Bray 1979). A more quantitative examination of the capacity of growth cones to exert tension was made by Lamoureux *et al* (1989) who attached the cell bodies of individual sensory neurons to the ends of glass needles and lifted them from the substratum allowing the still attached growth cones to exert a mechanical tension. The deflection of the glass needle provided a means of estimating that tension. As described earlier, tension in the growth cone results from contraction of the cortical actin meshwork.

How does tension in the growth cone lead to axonal elongation ? Bray (1991) has suggested that tension acts as a " second messenger ". That the adhesive forces binding a filopodium to the substrate somehow constrain actin mechanically and in so doing regulate assembly of the axon. Bray showed that when a growth cone is firmly fixed to a microelectrode and then towed passively over the surface of a culture dish by means of a motor, an axon with normal ultrastructure is produced (Bray 1984). Thus axonal growth is the result of an applied mechanical tension; substrate interactions and filopodial dynamics have no role. However there

is experimental evidence against Bray's model for axonal elongation based on the development of mechanical tension by the growth cone. Neurons growing on highly adhesive substrata such as polylysine do not show evidence of tension development, especially if serum is absent from the culture medium (Luduena 1973; Bray & Chapman 1985; Bray 1991). There is also evidence that neurons growing in tissue culture can extend processes similar in appearance to neurites in the presence of cytochalasin B (Marsh & Letourneau 1984; Letourneau *et al* 1987; McCaig 1989). These observations demonstrate that an organized actin cortex and tension development may not be essential requirements for growth of neuronal processes. It is possible that neurite growth under these conditions is a product of the artificial tissue culture environment. Bray has proposed that neurons growing on highly adhesive culture substrata such as polylysine exhibit abnormal morphologies with highly flattened growth cones, axons with irregular, wandering trajectories, and many lateral filopodia. Whereas neurites and growth cones *in vivo* appear much more like those growing on less adhesive culture substrates such as glass, on which tension development does occur. Bray goes on to argue that tension development, actin microfilaments and filopodial activity are not necessary to make neuronal processes, but are required to allow those processes to respond to extrinsic cues. However, McCaig (1989) showed that amphibian spinal cord neurons could extend neurites *in vitro* in the presence of cytochalasin D and that they could respond to a small applied electric field. Demonstrating that even in the absence of an organized actin skeleton and filopodial activity neurites may still be able to respond to some extrinsic guidance cues. He suggested that localized entry of Ca^{2+} into growth cones lacking filopodia may increase adhesion of the neurite to the substratum, and that locally increased adhesion accompanied by local

contraction of remaining actin filaments could enable the neurite to respond to guidance cues in much the same ways as normal.

An alternative to Bray's proposal that growth cone movement is linked to axonal elongation by the development of tension is Goldberg & Burmeister's view that axonal growth occurs in a sequence of three morphologically defined steps: protrusion, engorgement and consolidation. Firstly, filopodia and lamellipodia are protruded from the growth cone by a mechanism similar to that already described. These membrane protrusions are then engorged with cytoplasm, membrane organelles, and microtubules entering from the more distal regions of the growth cone. Movement of organelles is along microtubules and by Brownian motion. The engorged region of the growth cone then narrows and adjusts to the cylindrical shape of the neurite (Goldberg & Burmeister 1986, 1988, 1989; Burmeister & Goldberg 1988).

Goldberg & Burmeister believe that the function of filopodia in neurite growth is two fold: they explore the extracellular environment searching for guidance cues; and they provide the intracellular space needed for rapid engorgement. They suggest that as neurites are able to grow without filopodia the mechanical tension mechanism of neurite growth as proposed by Bray cannot be entirely correct. Working with *Aplysia* growth cones they identified a substratum-bound, growth-promoting factor that elicited its effect by promoting the engorgement step, rather than providing an adhesion against which filopodia can pull (Burmeister *et al* 1991). They suggested this was facilitated by localized weakening of the actin network. The application of computer-enhanced differential interference contrast video-microscopy to *Aplysia* growth cones allowed real time growth cone

dynamics to be investigated. Filopodial shortening necessary for Bray's tension model was never seen (Goldberg & Burmeister 1986).

The question of whether growth cones " pull " or " push " has been the centre of much debate in recent years (Bray 1987, 1991; Goldberg & Burmeister 1988, 1989; Turner & Flier 1989). Bray's observations on the effects of experimentally applied tension suggest a pulling mechanism. This hypothesis is supported by the work of Lamoureux *et al* 1989 and Heidemann *et al* 1990. However, Goldberg & Burmeister's experiments using high resolution microscopy suggest a pushing or stepwise protrusion, engorgement, consolidation mechanism. Experiments using cytochalasin B demonstrate that neurite-like processes can extend in the absence of filopodia and a functional actin-cortex capable of supporting traction (Marsh & Letourneau 1984; Letourneau *et al* 1987; Forscher & Smith 1988). These results argue against Bray's tension model. However, Bray himself states that filopodial activity and actin-based traction may only be necessary to allow growth cones to respond to environmental guidance cues and may not be required for neurite elongation as such. *In vitro* experiments on the effects of cytochalasin B have been carried out on glass or polypeptide substrates devoid of guidance cues. Its effects on neurites growing over extracellular matrix or other known guidance molecules remains to be tested.

The " push " versus " pull " debate is unresolved. The nature of the neurons used and the substratum on which they grow can influence growth cone behaviour and could help to explain some of the different interpretations of the models. Some of the experiments described in the present thesis are directed at this issue.

NEURONAL CULTURE

The use of neuronal cell and tissue culture has been central to the study of growth cone motilty, axonal elongation and the development of sensory innervation. Harrison (1907) was the first person to develop a successful technique for culturing neuronal tissue. He embedded explants of frog spinal cord in plasma clots bathed in a simple nutrient mixture and observed the subsequent growth of living nerve fibres through the clot matrix. He made the first direct observations of the highly dynamic protrusive activity of the growth cone and many of his ideas concerning the mechanism of neurite growth remain current.

Many major discoveries which have shaped the course of research in developmental neurobiology have arisen through cell culture. For example, the discovery and isolation of nerve growth factor (Levi-Montalcini *et al* 1954; Cohen *et al* 1954), the significance of the growth cone as an organelle of locomotion (Hughes 1953), and the importance of cell-substrate adhesion (Letourneau 1975a,b).

Many methods now exist for culture of a wide variety of neuronal types under a wide variety of experimental conditions. Plasma clots (difficult to prepare and offering little potential for detailed cellular and biochemical studies) were superceded by culture on two dimensional substrates (Levi-Montalcini & Angeletti 1963). Cultures of dissociated neurons were made by St. Amand & Tipton (1954) and dissociated sensory neurons by Nakai (1956). The advantages of culturing dissociated neurons on a 2-D substrate are immense. The properties and behaviour of individual cells can be analysed; biochemical dissection of cell-cell and cell-substrate

interactions becomes easier; and access for experimental manipulation is facilitated. During the last twenty years considerable progress has been made towards an understanding of how growth cones move and how their motility is related to axonal elongation and guidance. In 1975 by experiments Letourneau established the significance of cell-substrate adhesion in controlling neurite elongation *in vitro*. Many adhesive glycoproteins have since been isolated from extracellular matrix material and cell surfaces, and their role in controlling neurite outgrowth demonstrated by *in vitro* assay (Thiery *et al* 1977; Edelman 1984; Collins & Garrett 1980; Akers *et al* 1981; Rogers *et al* 1983).

Discerning the cues used by growing neurites to grow, stop growing, or change direction forms the basis of much current research in developmental neurobiology. Besides adhesive glycoproteins there are many other molecules that regulate neurite elongation, some operating by contact, others by long range diffusion. The identification of cue molecules depends on *in vitro* assays. The molecular mechanism whereby guidance molecules mediate growth cone behaviour must also be studied *in vitro*. *In vivo* systems containing multiple guidance cues, not all of which elicit outgrowth (some may inhibit), are too complex for us to distinguish among multiple mechanisms let alone understand how those mechanisms may operate. Tissue culture is therefore the principal method used in the present thesis.

CHAPTER 2

MATERIALS AND METHODS

Experimental Animals

Fertilized eggs of the chicken (*Gallus domesticus*) were obtained from either the Poultry Research Centre, Roslin, Edinburgh (strain J-Line), or Ross Breeders, Newbridge, Edinburgh (strain MPZ). Data from the two strains showed no systematic differences. The eggs were maintained in a humidified, force draft incubator at 37°C until they reached the required developmental stage. They could then be transferredto 12°C and maintained for up to 14 days without further development.

Dissection

Dissections were carried out using a trans-illuminated dissecting microscope in a tissue culture flow hood. A small window was first cut in the shell above the air sac. The chorio-allantoic membrane was then stripped off revealing the embryo, which was staged according to Hamburger & Hamilton (1956). Embryos of appropriate developmental stage were removed from the egg and placed in ice cold Trizma buffered physiological saline (TD; composition in mM : Na^+ , 137.5; K^+ , 5; Cl^- , 141.8; HPO_4^- , 0.7; trizma base, 24.7; pH 7.4), decapitated, cleaned of attached extra embryonic membranes and eviscerated.

Embryos were then transfered to a clean Sylgard (BDH) coated dish, immersed in TD and pinned dorsal surface up with the limb buds spread. At the early stages of development used, the developing skin and underlying tissues could be easily and cleanly removed to expose the neural tube and paired dorsal root ganglia (DRG) from cervical to lumbo-sacral segmental levels. Individual DRG were removed from the embryo with fine forceps using a plucking action. Ganglia from different segmental levels were easily identifiable. In all cases the isolated DRG were placed directly into ice cold TD and cleaned of nerve roots.

Culture of Floating DRG

A novel preparation was studied in this thesis. It was found by chance that DRG explants floating on the surface of serum-free culture medium sprouted extensive neurites within a few hours. For routine study culture at an air-liquid interface was achieved by placing an explanted ganglion in a small drop of buffer in an untreated 35 mm diameter plastic culture dish (Flow) and adding 2 ml of Ham's F-12 culture medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin sulphate and 100 μg/ml kanamycin sulphate and nerve growth factor (all from Sigma). Addition of the medium raised the ganglion to the surface. Alternatively, culture medium was added first and an isolated ganglion carefully placed onto the surface using a fine tipped pasteur pipette.

Floating ganglia cultured in the complete absence of Fetal Calf Serum (FCS) or Bovine Serum Albumin (BSA) were very sensitive to movement during the first few hours. The ganglia usually drifted to the edge of the culture dish where the optical properties made it difficult or impossible to

measure neurite outgrowth. Addition of 0.05% BSA (Sigma) to the cultures prevented the ganglia from drifting. Neurotrophic factor bioassays using floating and adherent ganglia (see below) were therefore carried out in medium containing 0.05% BSA.

Culture of adherent ganglia

Outgrowth from floating ganglia was compared with outgrowth from ganglia cultured on substrata coated with either poly-L-lysine (PLYS; Sigma) or polyornithine (PORN; Sigma) and laminin (BRL). PLYS substrata were prepared by incubating the culture wells for 3-4 h at 20°C with a 10 μg/ml solution in distilled water (Verna 1985). Dishes were then washed twice with distilled water and air dried. In some preliminary experiments flame sterilized 13 mm glass coverslips were coated as described. Data from these two protocols showed no systematic differences and PLYS coated plastic was used for all further experiments. PORN/laminin substrates were always prepared on 13 mm glass coverslips. These were first incubated for 30 min at 20°C with 50 μl of a 250 μg/ml solution of poly-DL-ornithine in sodium borate buffer (150 mM sodium tetraborate in distilled water; pH 8.4). PORN substrates increase subsequent laminin binding (Edgar *et al* 1984). Each coverslip was then washed three times with sterile distilled water and dried under a UV lamp for 60 min at 20°C. The coverslips were then incubated for 45 min with 50 μl of a 100 μg/ml solution of laminin in TD, and rinsed twice with TD. Laminin coated coverslips were always used on the day they were prepared and were never stored prior to use.

Freshly dissected ganglia were placed on the substratum and weighted with a small square of sterile dialysis tubing soaked in culture medium. Following incubation for 90 min at 37°C, 2 ml of culture medium was added and the dialysis tubing was then removed. This method facilitated adhesion of the ganglion to the substratum.

Co-culture of ganglia with target tissue explants

Limb bud ectoderm and mesenchyme were separated as follows. In younger embryos intact limb buds were dissected into 20 mM EDTA (Sigma) in TD, incubated for 30 min at 37°C, then washed three times with TD. Ectoderm was then stripped from the underlying tissue using fine forceps. In older embryos, the ectoderm could be removed from the the limb buds without EDTA treatment. Explants of tissue of approximately the same size as the DRG were then cut using forceps and transferred into ice cold TD.

Substrates were coated with Poly-L-lysine as described above. Isolated ganglia and target tissue explants were placed in close proximity (<1mm) on the substratum. A small square of dialysis tubing was laid over the explants for the first 90 minutes of culture to facilitate adhesion. These cultures were incubated in the complete absence of serum and nerve growth factor.

Assay of neurite outgrowth

Quantitative analysis of neurite outgrowth was carried out after 24 h of culture. The ganglia were examined using a Nikon Diaphot phase contrast

* A similar index taking into account both fibre density and fibre length was used by Landreth and Agranoff (1979) in their studies of retinal ganglion explants.

microscope. Images were captured using a SONY AVC D7CE video camera, contrast enhanced if necessary (Colorado Video, model 605) and printed using a Mitsubishi P66B video hardcopy unit. Photomontages were prepared for each ganglion. Each photomontage was then divided into eight equal sectors. The growth cone furthest from the edge of the explant was identified in each sector and connected by a straight line to the furthest growth cone in the two neighbouring sectors (Fig. 1). The area of the resulting octagon was measured using a digitizing tablet and software written for a BBC microcomputer (written by Dr. R.R. Ribchester). The area bounded by the edges of the explant was measured using the same program. Subtracting the area of the explant from the area of the octagon gave a value for the area of neurite outgrowth (mm^2). The density of outgrowth (neurites/mm^2) for each of the eight sectors was estimated subjectively on an integer scale from 0 to 5. These values were then averaged for each ganglion. A dimensionless index of neurite outgrowth was calculated by multiplying the average density by the total area covered by neurite outgrowth for each ganglion.*

When DRG were co-cultured with other explants it was necessary to determine whether outgrowth was directed. The centres of the DRG and adjacent explant were first connected with a straight line. A second line was then drawn through the centre of the DRG at 90 degrees to the first line, thus dividing the ganglion in half. Using these two lines as a starting point the ganglion was further divided into eight equal sectors as described above. The longest neurite was then measured in each sector and an average neurite length calculated for each half of the ganglion. Average estimates of neurite density were then made for each half ganglion and multiplied by the average neurite length to give a

dimensionless neurite outgrowth index. Comparison of the neurite outgrowth indices of the half of the ganglion proximal to the other explant and the distal half indicate whether the outgrowth of neurites is oriented by the presence of the adjacent explant.

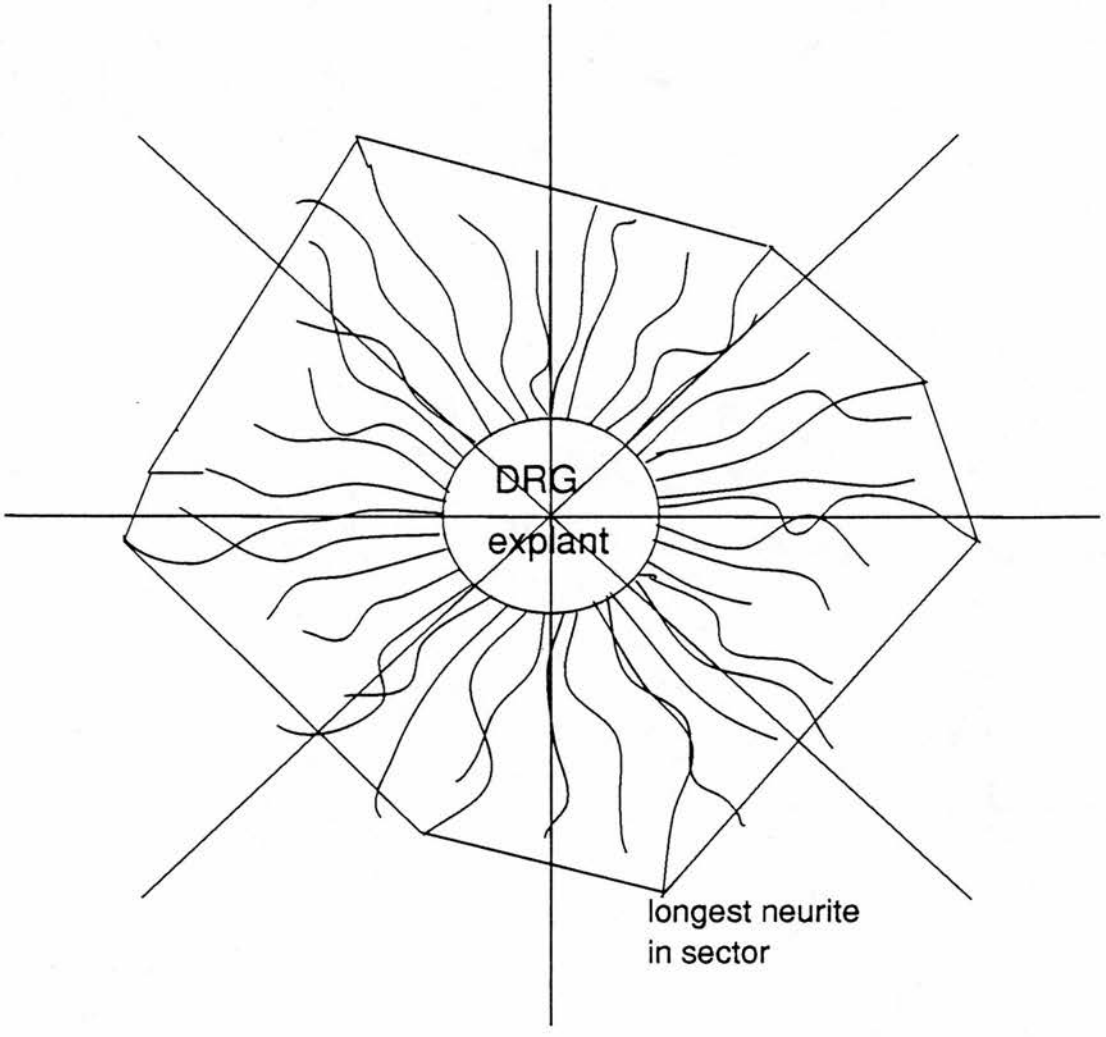


Fig. 1

Schematic illustration of the method used to determine the neurite outgrowth index.

Staining for the 68kD neurofilament subunit protein

Some floating ganglia were stained with antibodies against the 68 kD subunit of the neuron specific neurofilament protein (Sigma). A laminin coated coverslip was first positioned below the floating ganglion and then the culture medium was then carefully removed using a pasteur pipette. The ganglion was thus lowered onto the coverslip and was then incubated, in the absence of culture medium, at 37°C for 1 h to allow the ganglion to adhere. Ganglia were then stained with the antibody as follows:

1. Fix and permeabilize for 10 min in 100% methanol at -20°C.

2. Wash 10 min in phosphate buffered saline (PBS; pH 7.4).

3. Wash twice for 10 min in 0.05% Tween (a mild anionic detergent; Sigma) in PBS.

4. Wash twice for 10 min in PBS.

5. Incubate for 24 h in 68kD neurofilament antibody at 1:50 dilution in PBS at 4°C.

6. Wash twice for 10 min in PBS.

7. Incubate for 2 h in Fluorescein Isothiocyanate (FITC) conjugated goat anti-mouse IgG secondary antibody at 1:5 dilution in PBS at 37°C

8. Wash twice for 10 min in PBS.

9. Mount in aquapolymount on glass coverslips.

Fluorescence microscopy was carried out using a high pressure mercury light source and a blue (450-490 nm) excitation block (Nikon: B2-H) to view FITC conjugated antibody labelled cultures (peak emission 535 nm: green). Still photographs were taken on a 35 mm Nikon F-301 camera using Kodak Ektachrome 200 and 400 ASA film.

Preparation of colcemid, nocodazole and cytochalasin B

Stock solutions of nocodazole and cytochalasin B (Sigma) were prepared in dimethylsulfoxide (DMSO) and stored at 4°C prior to use (Bamburg *et al* 1986, Letourneau *et al* 1987). The colcemid (Sigma) stock solution was prepared in culture medium and stored at -20C prior to use (Yamada *et al* 1970, Bamburg *et al* 1986). Drugs were added to the cultures at the start of the incubation period to give final concetrations of 0.05-1.00 μg/ml cytochalasin B, 0.025-0.3 μg/ml colcemid, and 0.005-0.05 μg/ml nocodazole. Control cultures received DMSO or culture medium.

Preparation of conditioned medium

Ectoderm was stripped from st.* 25 limb buds as described previously, transferred into a 0.01% solution of trypsin (Sigma) in TD and incubated at 37°C for 30 min. The tissue was then spun down (approx. 6 sec at 14,000 rpm, Eppendorf microfuge) and resuspended in TD containing 0.025% soybean trypsin inhibitor (Sigma) and 0.02% DNase 1 (Sigma) which denatures DNA released from lysed cells (DNA may otherwise have combined with trypsin to form a gel resulting in cell re-aggregation). The

*stage

tissue was then triturated by no more than 10 passages through a fire-polished pasteur pipette to give a single cell suspension. This was again spun down (6-10 sec at14,000 rpm) and resuspended in Ham's F-12 culture medium containing antibiotics, L-glutamine and 10% Fetal Calf Serum (Flow). The cell density was determined using a haemocytometer and adjusted to approx. $1.2x10^6$ /ml. 1 ml of the resulting cell suspension was added to 35 mm diameter tissue culture wells pre-coated with PLYS. The cultures were then incubated at 37°C for 1 h to allow cells to adhere. A further 1 ml of pre-incubated medium supplemented with FCS was then added to each well to give a final volume of 2 ml. After 24 h of culture this medium was removed and the cultures washed twice with pre-incubated serum-free culture medium. 2 ml of serum-free culture medium was then added to each well and the dishes were returned to the incubator. After 48 h, medium was removed from each well and used as conditioned medium to culture DRG explants.

Statistical tests

A Wilcoxon unpaired non-parametric test was used to compare the mean neurite outgrowth index under different conditions.

CHAPTER 3

INTRODUCTION

Neural tissue explants are often used as an adjunct to dissociated cell preparations for studies of axonal outgrowth, guidance and the formation and maintenance of neural connections (Lumsden & Davies 1983; Ebendal 1989; Stoppin *et al* 1991; Tuttle & Matthew 1991). For instance, the extended family of neurotrophin growth factors was identified using techniques for bioassay of growth factor activity on neural explants and dissociated neurons. The quantitative effects of neurotrophic factors in regulating neurite outgrowth have been measured using a variety of different methods, from outgrowth of dissociated neurons adhered to coated substrata, to assays in three-dimensional collagen gels. These kinds of studies show that the amount and extent of fibre outgrowth depend not only on the concentration of soluble growth factors, but also on the composition of the substrate. Many molecules routinely used to coat culture substrata evidently have independent, co-trophic effects on neurite outgrowth. When trying to characterize neurotrophic activity, it would be helpful if the effects of soluble and substrate bound activities could be distinguished.

This chapter describes the growth of neurites from dorsal root ganglion explants floated at the air-liquid interface of culture medium. This preparation offers an ideal method for routine bioassay of neurotrophic activity, which is not influenced by macromolecular substrates or matrices.

In addition, the finding that neurites will grow along an air-liquid interface raises issues concerning the roles of cell adhesion and cellular traction in the mechanism of axonal growth.

RESULTS

Appearance of floating ganglion cultures

A raft of extensive neurite outgrowth projected from dorsal root ganglia within 24 h of culture at the air-liquid interface in serum-free medium (Fig.3.1). Typically, neurites emerged from the explant in thick bundles which gradually became less fasciculated (Fig.3.2), although the complete absence of fasciculation was rare even for neurites which had extended the furthest. Growth cones were clearly visible at the tips of the neurites, and their activity, including formation of lamellae and filopodia (microspikes) could be seen in real time (Fig.3.3). Staining cultures with fluorescently labelled antibodies against the 68 kD neurofilament protein confirmed that the cellular processes growing from the floating ganglia were neurites (Fig.3.4).

The pattern of outgrowth around ganglia was variable. Some ganglia only extended neurites from one side, others showed bias towards one side or another, many had patchy outgrowth, and some produced an even halo of outgrowth similar to that seen from adherent ganglia (Levi-Montalcini *et al* 1954). Growing neurites appeared to elongate along the underside of the surface of the medium and were clearly attached to the interface at the

growth cone. Neurites were commonly curved or bent, appearing similar to those growing on adhesive substrates such as polylysine (Bray 1979, 1991) and were rarely straight, suggesting that they were attached to the interface all along their length as well as at the growth cone. In addition to neurite outgrowth, non-neuronal cells migrated from the explants up to and beyond the tips of the longest neurites. Non-neuronal cells did not appear to be associated with either the extent or direction of neurite growth. Nor were non-neuronal cells ever seen to be attached or adhered to neuronal growth cones.

The effect of serum on neurite outgrowth

Neurite outgrowth did not occur from the floating explants when Fetal Calf Serum (10%) was included in the culture medium. This was unexpected as serum is normally added to cultures of neuronal cells or explants without detrimental effects on neurite outgrowth. Despite many decades of use in cell and tissue culture, the exact composition of serum remains unknown. Inhibition of outgrowth was mimicked by serum albumin alone. Increasing the concentration of BSA in the culture medium systematically reduced the amount of neurite outgrowth from the floating ganglia. For example, in medium containing 10% BSA neurite outgrowth was dramatically inhibited (Fig.3.5) and the neurite outgrowth index reduced to about a fifth of that in serum free medium (Fig.3.6). This effect may be attributable to the reduced surface tension of medium containing serum or albumin (see Discussion).

Fig.3.1

Neurite outgrowth after 24 h of culture from dorsal root ganglion floating at the surface of serum-free culture medium containing 20 ng/ml NGF. Calibration bar, 1 mm.

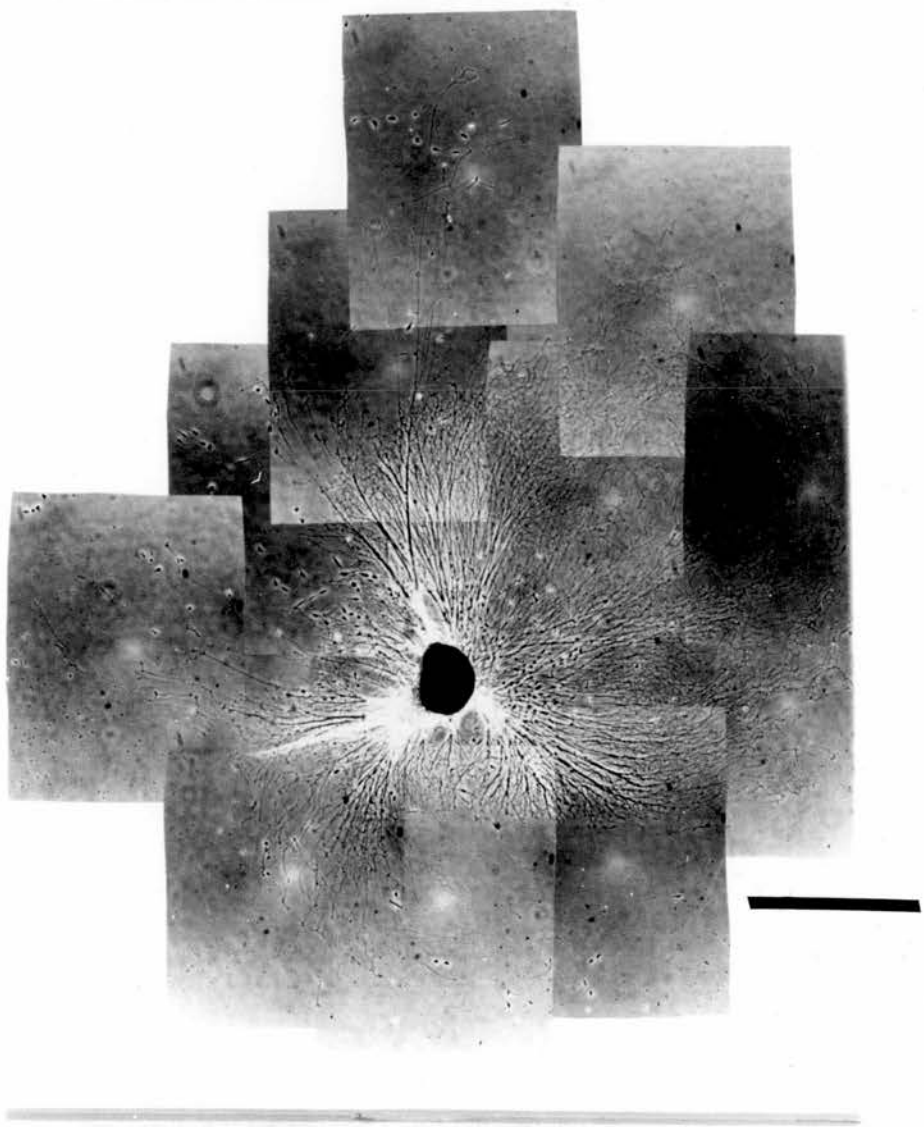


Fig.3.2

Neurites projecting from a floating DRG viewed under Hoffman optics to highlight fasciculation. Calibration bar, 200 μm.

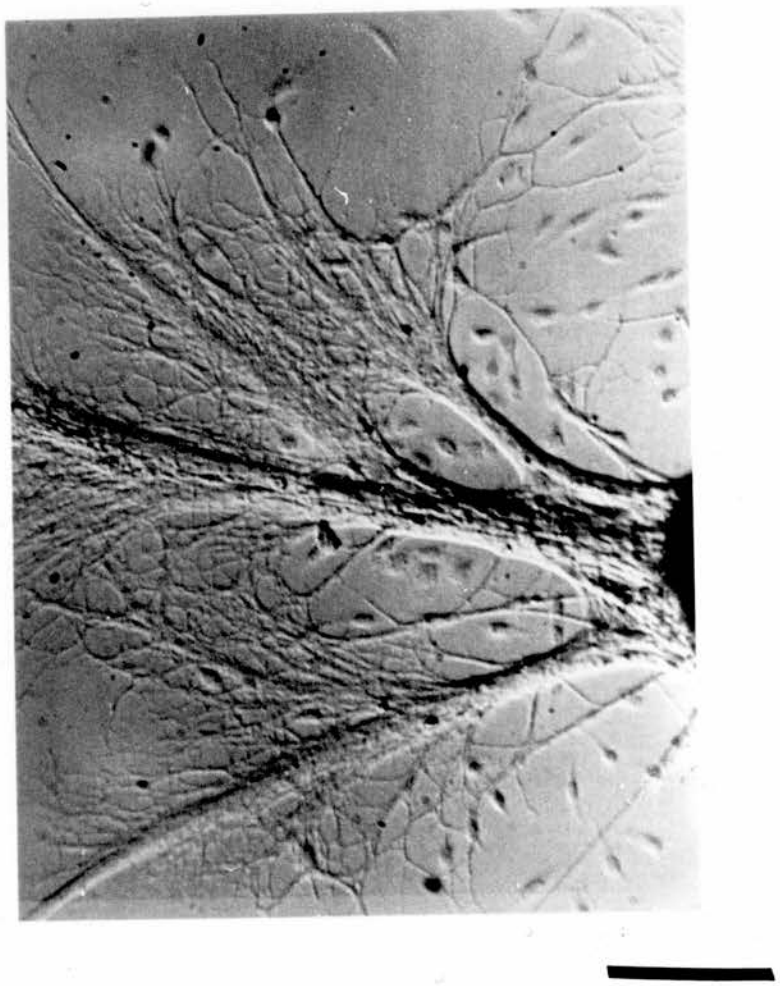
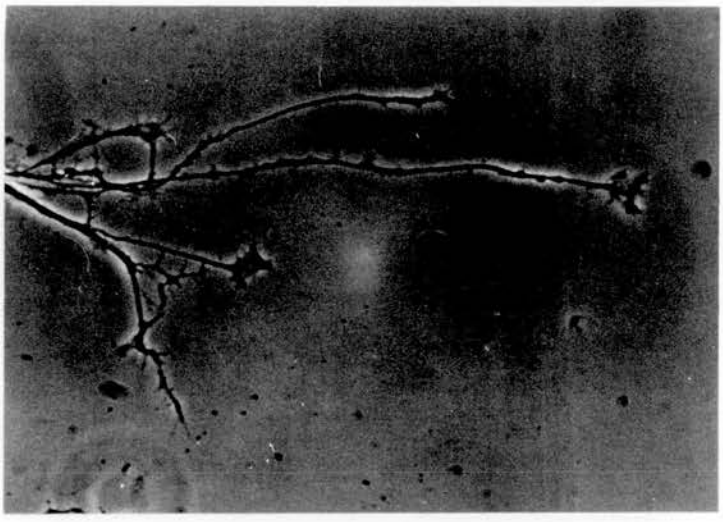
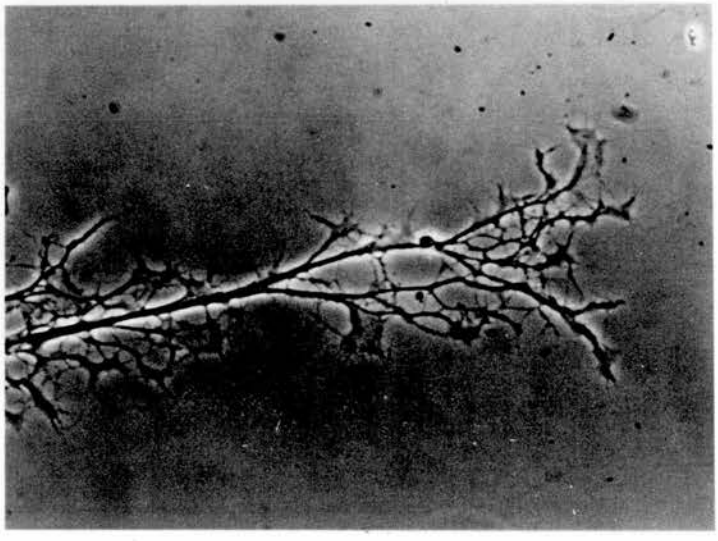


Fig.3.3

Higher power view of neurites tipped with growth cones projecting along the underside of serum-free culture medium. Calibration bar, 200 μm.





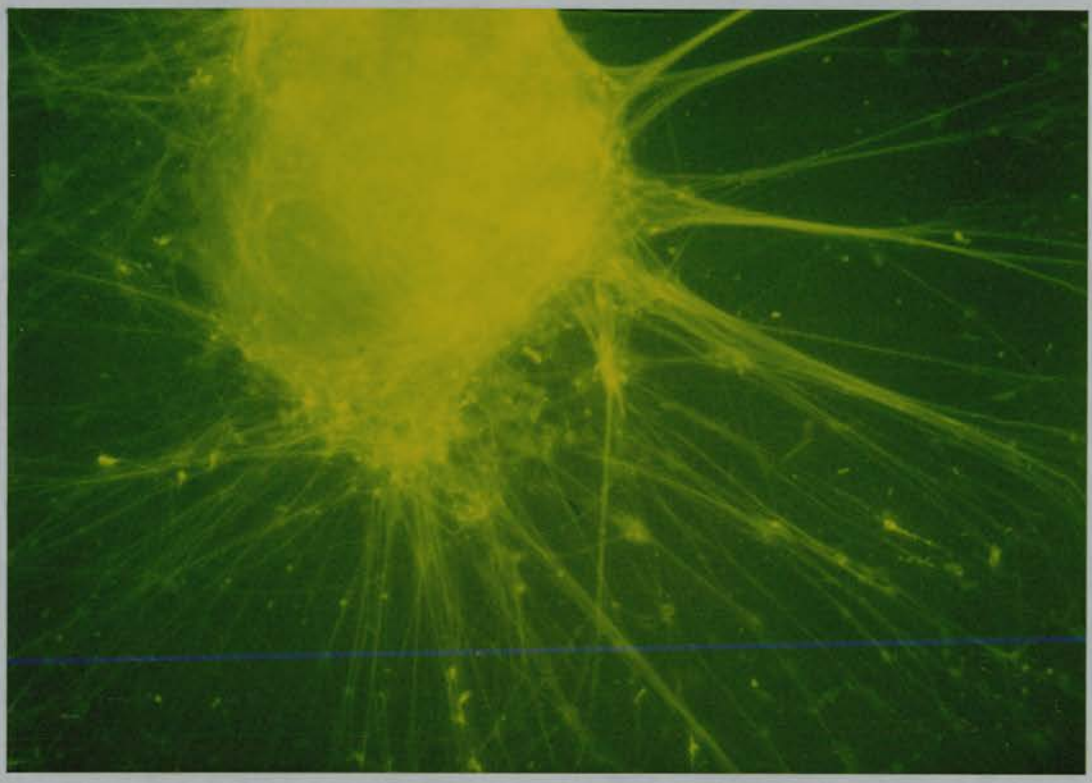
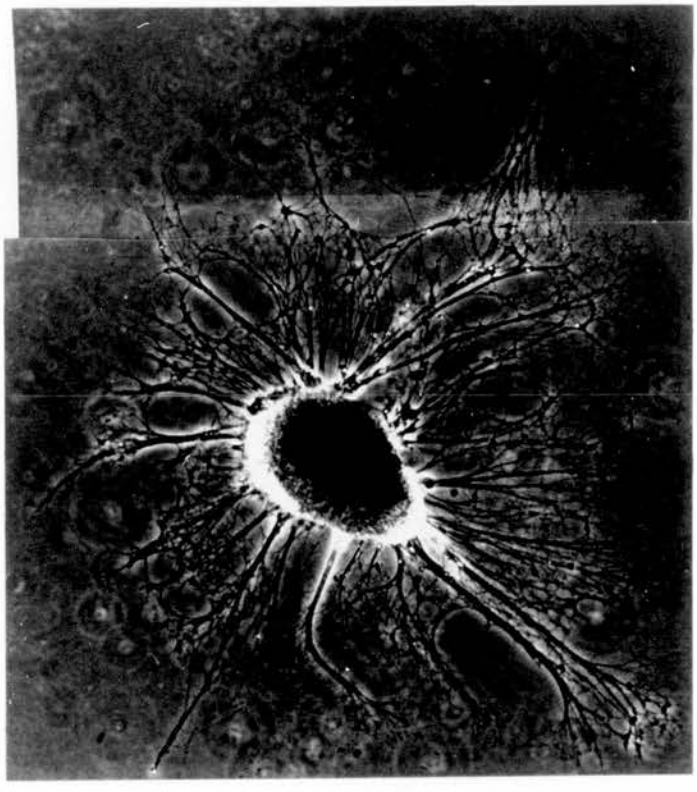


Fig.3.4

Neurites from floating ganglia stained with the anti-68 kD neurofilament subunit antibody.

Fig.3.5

Neurite outgrowth after 24 h of culture from dorsal root ganglia floating on the surface of medium containing 10% bovine serum albumin and 20 ng/ml NGF. Calibration bar, 1 mm.



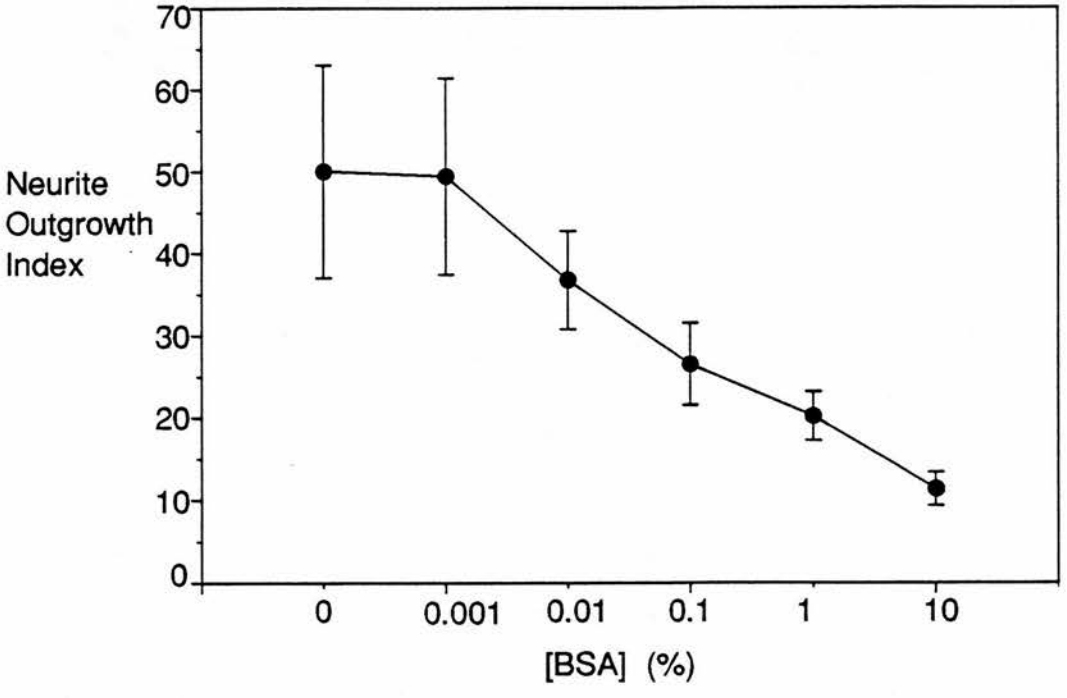


Fig.3.6

Neurite outgrowth after 24 h in medium containing bovine serum albumin and 20 ng/ml NGF. Each point shows the mean±S.D. obtained from between six and ten cultures.

Bioassay of NGF activity

In order to test the potential utility of the floating DRG preparation as a bioassay for neurotrophic factors, isolated ganglia were cultured in media containing different concentrations of NGF. Outgrowth of neurites was then compared with that from ganglia adhered to either PLYS or PORN/laminin. A small amount of BSA was included in the culture medium. This prevented floating ganglia from drifting (see Methods).

Outgrowth of neurites from the floating ganglia was strongly dependent on the concentration of NGF in the medium (Figs.3.7 and 3.8), with an apparent ED50 of 0.60 ng/ml (Fig.3.9A). Less outgrowth was seen at an NGF concentration of 50 ng/ml than at 5 ng/ml however (see Discussion).

Neurite outgrowth from ganglia adhered to a PORN and laminin coated substrate was greater than that from floating DRG over a range of NGF concentrations from 0.05-50 ng/ml (Figs.3.10A and 3.9B). A substrate coated with PLYS alone was considerably less effective in promoting neurite outgrowth (Figs.3.10B and 3.9C). Uncoated tissue culture plastic was the least effective substrate for neurite outgrowth (Fig.3.11).

On laminin and on PLYS very few of the neurites appeared to be fasciculated, in contrast to the plethora of neurite fascicles generated by floating DRG. Outgrowth from adherent ganglia was always radial in contrast to the frequently biased or patchy outgrowth seen from floating ganglia, and migration of non-neuronal cells was very limited (Fig.3.10).

A small amount of outgrowth was seen on PORN/laminin even in the complete absence of NGF. The mean neurite outgrowth index of 4.03±1.19 (mean±S.D.; n=6) was significantly different from that on PLYS (0.31± 0.05; n=7; $p<0.05$) or at the surface of the culture medium (0.67±0.52; n=6; $p<0.05$; Fig.3.12). This suggests laminin is capable of stimulating a significant amount of neurite outgrowth from E 7 DRG even in the absence of soluble neurotrophic factors or serum. The amount of neurite outgrowth from floating ganglia cultured in the absence of NGF was not significantly different to that from ganglia adhered to PLYS ($p>0.05$).

Fig.3.7

Neurite outgrowth after 24 h of culture in medium containing 5 ng/ml NGF and 0.05% BSA. Calibration bar, 1 mm.

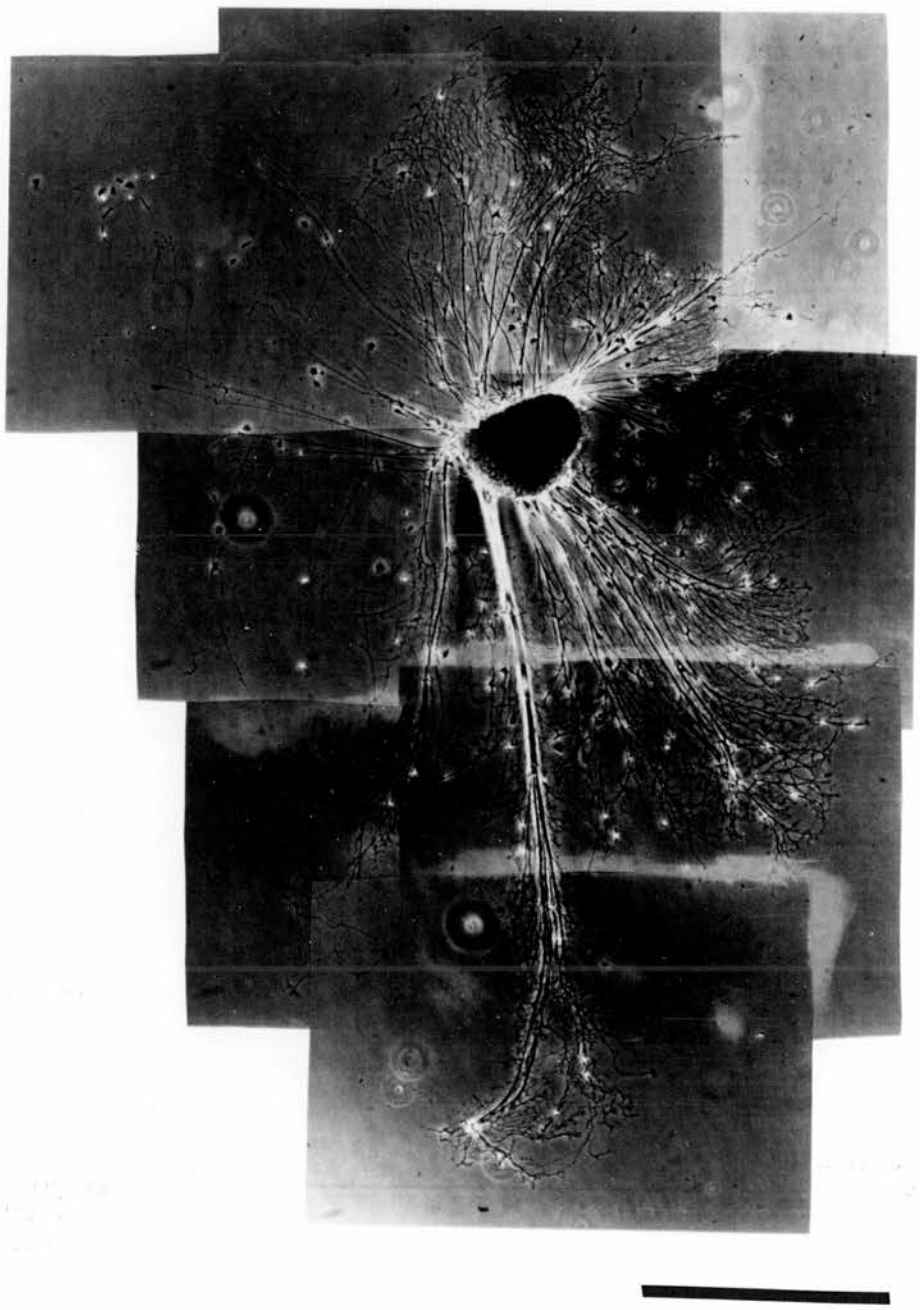
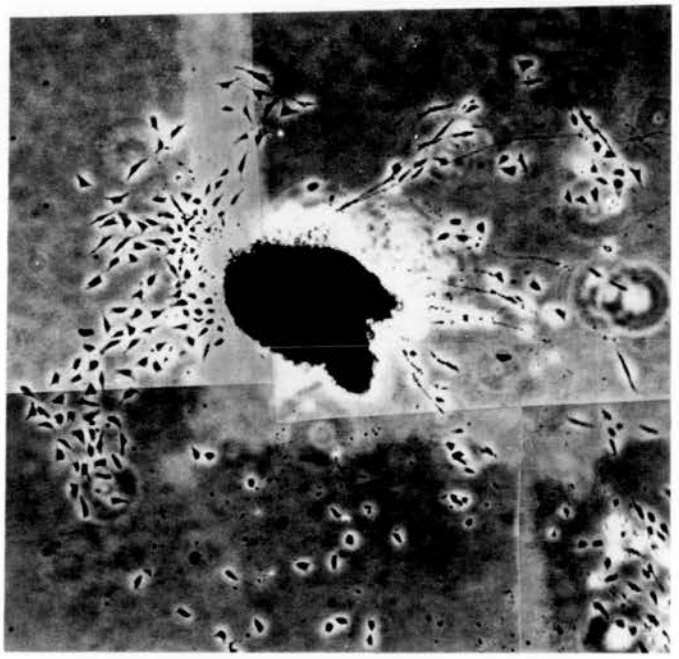


Fig.3.8

Neurite outgrowth after 24 h of culture in medium containing zero NGF and 0.05% BSA. Calibration bar, 1 mm.



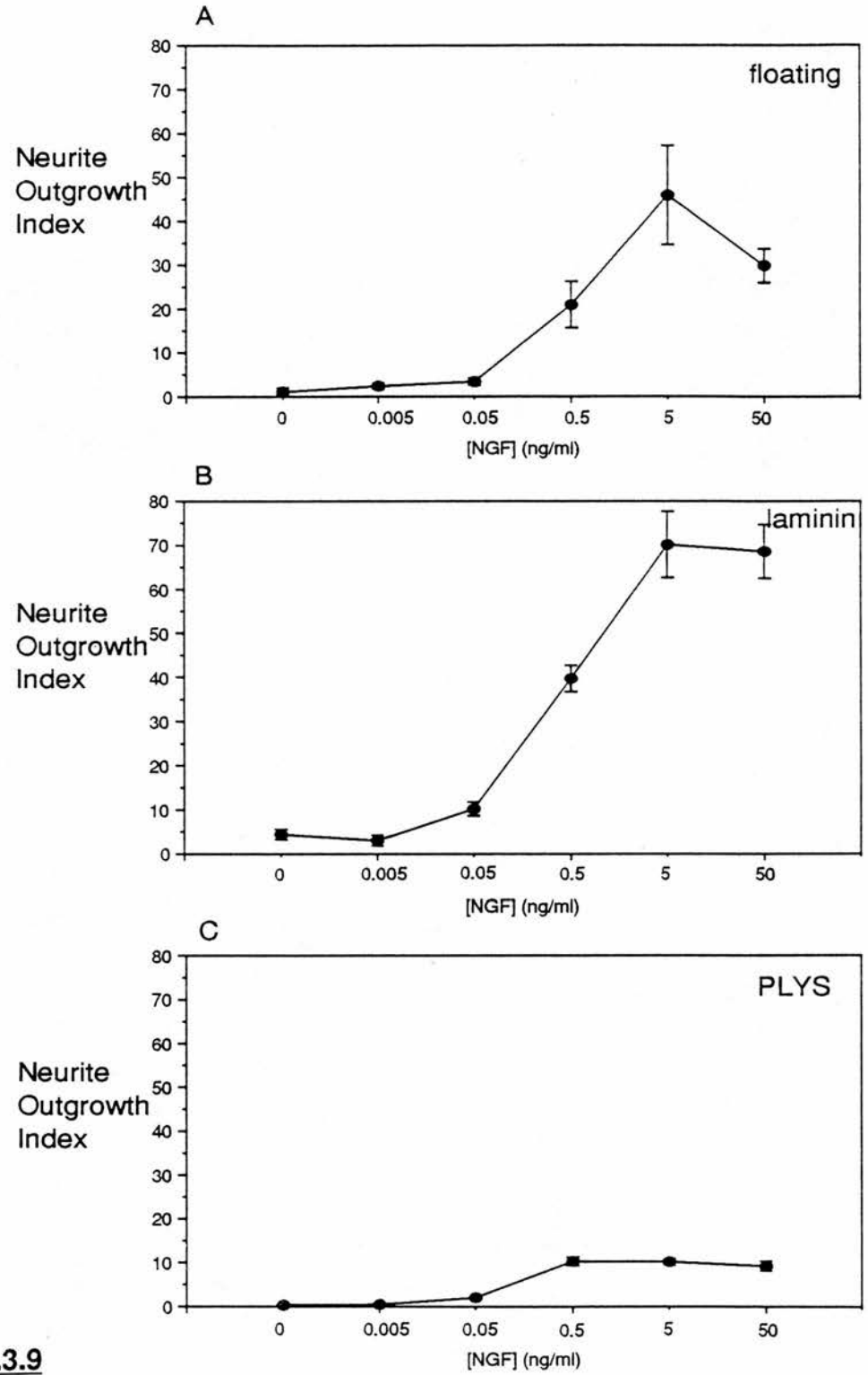


Fig.3.9

Dependence of neurite outgrowth on NGF concentration. Neurite outgrowth was assayed after 24 h of culture in medium containing 0.05% BSA. The mean±S.D. from between six and ten cultures is shown for each point. A, ganglia suspended at an air-fluid interface. B, ganglia adhered to a PORN and laminin coated substrate. C, ganglia adhered to a PLYS coated substrate.

Fig.3.10

Neurite outgrowth after 24 h of culture in medium containing 5 ng/ml NGF and 0.05% BSA. A, on a PORN and laminin coated substrate. B, on a PLYS coated substrate. Calibration bar, 1 mm.

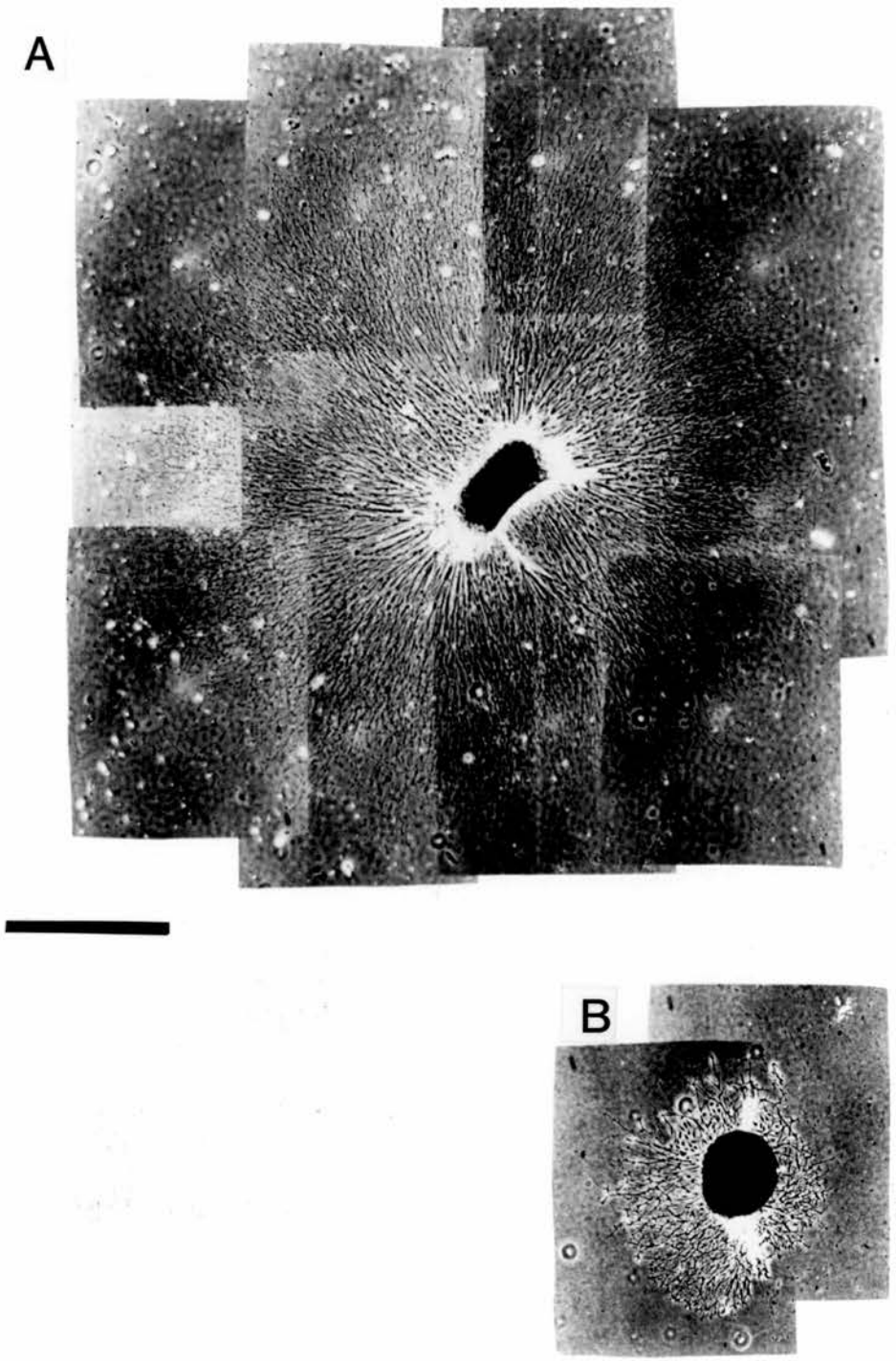
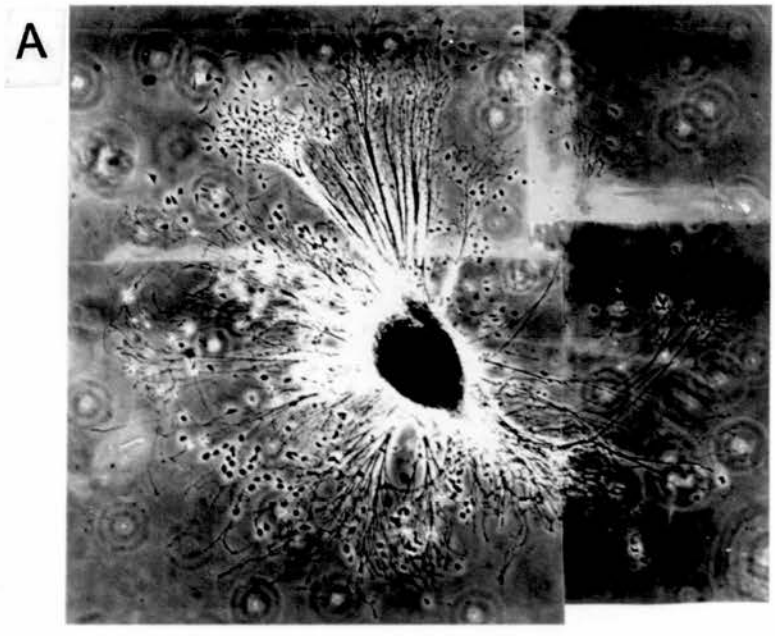
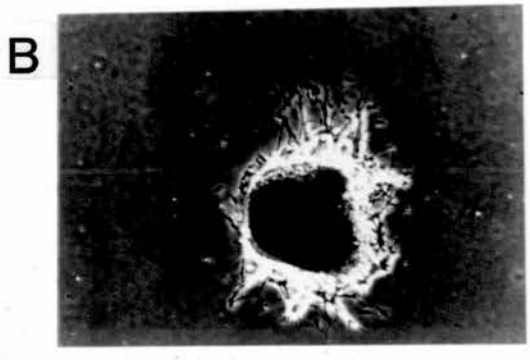

A
B

Fig.3.11

Neurite outgrowth after 24 h of culture in medium containing 5 ng/ml NGF and 0.05% BSA. A, floating at the interface; B, on tissue culture plastic. Calibration bar, 1 mm.


A


B

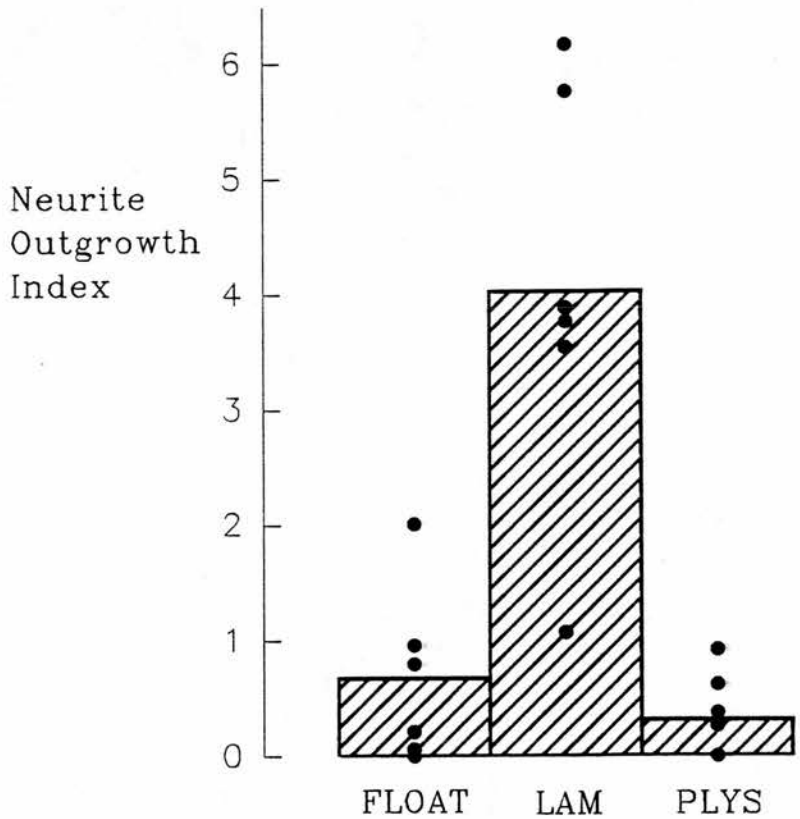


Fig.3.12

Neurite outgrowth after 24 h of culture in medium containing zero NGF and 0.05% BSA. Bar height represents the mean of the explant scores, and data points are also shown.

DISCUSSION

The main findings of this chapter are first, that dorsal root ganglia from chick embryos extend neurites when floating at the surface of culture medium, provided serum is absent or reduced in concentration in the medium. Second, neurites tended to fasciculate more readily during outgrowth from floating DRG, compared with ganglia adhered to solid substrata. Third, the amount and extent of neurite outgrowth depends on the concentration of NGF in the culture medium.

The behaviour of neuronal cells and tissues has been extensively studied in culture on a variety of substrates, from glass or plastic coated with adhesive molecules to three dimensional collagen or agar matrices (for example Letourneau 1975a,b; Rogers *et al* 1983; Rutishauser & Edelman 1980; Ebendal 1989). Some types of eukaryotic cells have been cultured on fluid substrates, inert hydrophobic liquids such as fluorocarbons that have a density greater than that of the aqueous medium (Giaever & Keese 1983). However, growth of neurons on a fluid substrate has not been demonstrated. Adhesion and proliferation of bacteria is known to occur at an air-liquid interface (Marshall 1980) but this has not been described for mammalian cells. The finding that neuronal processes will grow at an air-liquid interface was therefore most unexpected.

How are neurites able to extend and elongate from floating ganglia along the surface of culture medium? It has long been recognized that adhesion between the surface of the growth cone and its substratum is important in neurite extension (Harrison 1907, 1910; Letourneau 1975a). As the tip of

the neurite extends the protruded filopodia must adhere to the substratum or the extension could be transient and fold back on itself. If, however, the adhesions formed were irreversible they could inhibit forward movement altogether. Thus neurite elongation depends on cycles of adhesion and de-adhesion. The main effect of adhesion is that it permits traction, the principle mechanism of cell locomotion (Bray 1982, 1991; Singer & Kupfer 1986). Bearing in mind the fundamental importance of growth cone-substratum adhesion in neurite elongation on solid substrata, it is likely that extension of neurites from ganglia floating at the surface of serum-free culture medium also involves adhesive interactions. The manner in which adhesion between the cell surface and an air-liquid interface is brought about is not known. However, discussion of the proposed theories of cell adhesion may be useful for establishing the types of adhesive interactions which may operate at an air-liquid interface. It should be noted that elongation of neurite-like processes has been reported in the absence of substrate adhesion. In the presence of high concentrations of taxol, an agent that stimulates microtubule assembly, and cytochalasin B, which depolymerizes actin, neuronal processes grow through the culture fluid (Letourneau *et al* 1987). However, neurites formed in this environment are abnormal in morphology and ultrastructure.

Growth cone-substratum adhesion

There is no clear answer to the question of how cells adhere, either to each other or to extracellular substrata. Adhesion of cells to an air-liquid interface has not been studied and remains particularly obscure. It is thought that two types of interaction contribute to cell adhesion: physicochemical interactions depending on the properties of the cell

surface, and more specific receptor-ligand type molecular interactions (Grinnell 1978; Lackie 1986; Turner & Flier 1989). The following section considers some of the evidence supporting each of these theories of adhesion, and relates these models to the present findings.

Physicochemical forces contributing to cell adhesion are primarily an expression of surface charge. Cells have a negative surface charge (Heard *et al* 1961; Curtis 1967; Mehrishi 1972) and electrostatic repulsive forces exist between cells. There are also forces of attraction based on the London-van der Waals dispersion forces which act between materials of similar composition. Such forces could exist between cells due to the same basic composition of plasma membrane (Lackie 1986). The relative strengths of attraction and repulsion decline with distance but follow different power laws. The balance between forces varies with distance and two zones exist at which attraction exceeds repulsion (Derjaguin & Landau 1941; Curtis 1967). These zones are calculated for cells to be less than 2nm and between 15-25 nm (Pethica 1961; Curtis 1967). These distances are similar to the separations seen in tight junctions (2 nm) and non-specialized apposition of cell surfaces(15-25 nm).

The adhesion of cells to glass or plastic culture substrata is also influenced by the substrate charge. Rappaport (1971) showed that a critical number of negative charges on glass substrata was required for cell adhesion. Maroudas (1975,1977) found that by depositing a layer of sulphonate ions onto a plastic surface by treatment with sulphuric acid, untreated plastic could be made to support cell adhesion. He then calculated the optimum amount of negative charge (sulphate ion in this case) a surface would need to carry to facilitate adhesion. However,

further work by Curtis *et al* (1983, 1986) demonstrated that treatment with sulphuric acid deposits only a minimal amount of sulphate ion, insufficient to allow adhesion. They showed that exposure to sulphuric acid generates high surface densities of hydroxyl groups. Blocking these groups via acetylation inhibited the adhesion of fibroblasts, demonstrating that hydroxyl groups are important for adhesion to treated plastic substrata. They also suggested that adhesion to plastic may, at least in part, be mediated by formation of hydrogen bonds between cell surface carbohydrates and the hydroxyl groups on the substratum.

In addition to adhesion, cell locomotion may also depend on substrate charge. Sugimoto & Hagiwara (1979) and Sugimoto (1981) studied fibroblast locomotion on substrates of differing surface charge and found that locomotion speeds increased with increasing negativity of the substrate charge. The growth of dissociated sensory neurons has also been shown to be influenced by the net negative charge on the substrate with preferential growth on more negatively charged surfaces (Torimitsu & Kawana 1990).

During development many adhesion-dependent processes occur: cell condensation, tissue aggregation, movement of groups or sheets of cells, and migration of single cells are all essential features of morphogenesis. Clearly there is great specificity of cell adhesion which is difficult to explain by the kinds of physical mechanisms described above. Adhesion involving some form of receptor-ligand interaction is the alternative to a purely physicochemical explanation of cell adhesion.

Many molecules have now been identified which promote adhesion by a specific receptor mediated mechanism. For example fibronectin, a component of the extracellular matrix also found in serum, promotes adhesion of a wide variety of cell types from fibroblasts and epithelial cells to neurons (Grinnell 1978; Klebe *et al* 1987; Rogers *et al* 1983). Other matrix molecules such as laminin, collagen and heparin sulphate proteoglycan may also promote cell adhesion (Hay 1981; Sanes 1989). Tissue-specific cell adhesion molecules (CAM's) have also been discovered. Temporal and spacial expression of CAM's, extracellular matrix molecules and their receptors could account for the specific adhesive phenomena and complex morphogenetic movements which occur during embryogenesis (Edelman 1986; Sanes 1989).

Specific protein interactions may also facilitate adhesion to culture substrata. Adsorption of protein from solution onto glass or plastic is extremely rapid (Olsen & Kletschka 1973), enabling substrata to be experimentally coated with adhesive molecules. Adhesion of cells then proceeds via the same receptor-ligand interactions that mediate adhesion *in vivo*.

If culture medium contains serum, the substrate will become coated by a layer of adsorbed proteins (Grinnell 1978). Serum contains the adhesive glycoprotein fibronectin, as well as a number of other known adhesive molecules (vitronectin, fibrinogen) and possibly others, as yet unidentified (Yamada 1983; Curtis & Forrester 1984). Cell adhesion may be mediated by how the cells interact with these adhesive molecules. However, serum also contains glycoproteins such as alpha-1-antitrypsin and alpha-2-macroglobulin which inhibit cell adhesion (Curtis & Forrester 1984). Thus

adhesion of cells to glass or plastic in the presence of serum is mediated by a complex set of interactions involving adhesive and adhesion-inhibiting glycoproteins as well as physicochemical properties of the substrate such as surface charge and degree of hydroxylation.

A further finding pertinent to cell adhesion in the presence or absence of serum is that cells in culture frequently and rapidly secrete proteins which adsorb to the substrate, and may aid adhesion. For example, fibroblasts in culture secrete fibronectin (Grinnell 1978).

To summarize at this point, physicochemical theories and receptor mediated mechanisms have been proposed to explain cell-cell and cell-substratum adhesion. Physical theories are concerned primarily with electrostatic interactions, adsorption, and hydrogen bonding and there is some supportive evidence (Curtis 1973, 1983; Maroudas 1975, 1977; Grinnell 1978). These theories accurately predict the dimensions of cell junctions *in vivo*, but are rather unspecific and cannot explain morphogenetic processes (Edelman 1986). The receptor-ligand theory describes adhesion as the consequence of specific molecular interactions between cell surface receptors and molecules in the extracellular environment. Many adhesive proteins have been identified, from secreted proteoglycans to CAM's, and their temporal and spatial expression during development could more readily explain morphogenesis (Edelman 1986; Sanes 1989; Rutishauser & Jessell 1988). Lackie (1986) and Grinnell (1978) suggest that both physicochemical and specific molecular interactions contribute to adhesion, with certain cell types or cells at particular stages of development relying more on one type than the other.

Experimental investigation of the mechanism of adhesion at an air-liquid interface does not form part of this thesis. However, something of the nature of the adhesive interaction can be inferred from the finding that optimal neurite outgrowth occurs in serum-free medium.

Adhesion of dissociated sensory neurons to tissue culture plastic increases in serum-free medium (Luduena 1973). The cell bodies appear flattened onto the substratum and neurites are usually curved or bent suggesting they are adhered all along their length. This may be due to the absence of adhesion inhibiting proteins (see above). In contrast, when neurons are cultured in serum-containing medium neurites are straight and appear to be attached to the substrate only at the growth cone (Luduena 1973; Bray 1979; Shaw & Bray 1978). Bray (1979) states that since neurites attached only at their cell body and growth cone are straight this is evidence that they are under tension and consequently that growth cones are exerting traction. In the present study, neurites were cultured in serum free medium and were generally curved or bent. This suggests strong adhesion between the neuronal cell surface and air-liquid interface along the length of the neurite, and lack of tension development.

The results presented in this thesis also show that in serum free culture medium the appearance of neurites growing from floating ganglia is similar to that of neurites elongating on polylysine (PLYS). However when serum or serum albumin is present in the culture medium, neurites growing at an air-liquid interface and on PLYS behave in opposite ways. Neurites attached to PLYS continue to grow rapidly while those at an air-liquid interface are dramatically inhibited.

Neuronal adhesion and neurite elongation on solid substrates is thought to be mediated by specific glycoproteins adsorbed to the culture substrate. This is reflected by the finding that very little neurite outgrowth occurs from DRG adhered to tissue culture plastic in serum-free medium. In contrast, neurite outgrowth from floating ganglia is most prolific when serum is completely absent from the medium. Clearly a layer of adsorbed serum protein does not mediate adhesion at an air-liquid interface. However, adhesive glycoproteins are often secreted by neurons, glial cells, or fibroblasts and could facilitate adhesion (Grinnell 1978).

Evidence against proteins adsorbed at an air-liquid interface mediating adhesion comes from conformational studies. Adsorption of fibronectin, and other matrix molecules which may enhance neurite growth, at air-liquid interfaces has not been investigated. However, studies of the adsorption of enzymes and serum albumin at air-liquid interfaces have been undertaken (MacRitchie 1987; Graham & Phillips 1979a,b). Conformational changes associated with adsorption are considerable and may result in the complete unfolding of the molecule into an extended chain, often accompanied by loss of biological activity (Graham & Phillips 1979b). It is possible that adhesive glycoproteins secreted by cells in the ganglion would be inactive if adsorbed at the interface, although their contribution to growth cone adhesion cannot be ruled out.

In addition, the dramatic difference in neurite outgrowth from floating ganglia compared with outgrowth on tissue culture plastic suggests that adhesion and elongation at an air-liquid interface are not mediated by an adsorbed layer of secreted protein.

One of the major functions of cell-substrate adhesion is to allow tension development. Monolayers of adsorbed molecules at air-liquid interfaces are visco-elastic and flow rates can be measured (Moore & Eyring 1938). However, tension development demands an immovable substrate against which to pull, and it is uncertain whether a viscoelastic molecular monolayer could fulfil this demand. Lack of tension development is supported by the present finding that neurites are usually curved or bent in appearance (see Bray 1991; chapter 4 of this thesis).

If a layer of adsorbed protein does not mediate adhesion and neurite elongation at an air-liquid interface, it is likely that some form of physicochemical interaction is facilitating outgrowth. Culture medium contains a variety of ions as well as polar molecules such as amino acids and will therefore possess a surface potential. This potential may be due to electrical double layers set up by the orientation of the molecules adsorbed, orientation of the solvent molecules, distribution of ions, or to all these causes (Adam 1938; Davies & Rideal 1961). The cell surface also bears an electric charge (Curtis 1967) so it is possible that adhesion between cells and an air-liquid interface is primarily the result of an electrostatic interaction.

Increasing the concentration of serum albumin in the culture medium reduces the surface tension (as demonstrated by a simple capillarity test) and affects the surface potential (Graham & Phillips 1979a). The observed decrease in neurite outgrowth in high concentrations of serum albumin could be caused by a change in the electrostatic interaction between the growth cone and interface. The growth of sensory neurites has been shown to be influenced by substrate charge (Torimitsu & Kanawa 1990).

In addition to its effects on surface tension and surface charge BSA could inhibit neurite outgrowth by non-specific binding to growth factor receptors or ion channels. However, whole serum also inhibits neurite outgrowth at an air-liquid interface suggesting that inhibition of neurite extension results from changes in the properties of the interface.

Neurite fasciculation

Selective axonal fasciculation results in formation of specific nerve tracts during development and is also a feature of neurite outgrowth *in vitro*. The present study shows that neurites growing from floating ganglia fasciculate more readily than those adhered to solid substrates. Fasciculation is thought to be mediated by adhesive glycoproteins, such as NCAM, L1 and contactin on the axonal cell surface (see Rutishauser & Jessell 1988, for review) and has been shown *in vitro* to be affected by the composition of the culture substrate. Neurites on less adhesive substrates may exhibit more pronounced fasciculation. The finding that neurite fasciculation is greater at an air-liquid interface than on PLYS or laminin coated substrates suggests that the air-liquid interface may be less adhesive. However, neurites growing from floating ganglia are often curved suggesting a high degree of adhesion to the interface. In addition, cytochalasin B does not inhibit neurite elongation from floating ganglia and this is further evidence for a strong adhesive interaction between the neurites and interface (see chapter 4). Why do neurites elongating at an air-liquid interface show significantly greater fasciculation than those adhered to PLYS or laminin coated substrates? Usually when neurites fasciculate in culture it is because the surfaces of other neurites are more effective substrates for growth. However, this may not be the case when

neurites elongate at the interface of culture medium. In this system fascicles are thick near the edge of the explant with neurites and growth cones furthest from the edge of the explant showing much less fasciculation. If the surfaces of other cells was a preferred growth substrate then it would be expected that the degree of fasciculation would remain fairly constant along the length of the growing neurites. As this is not seen, fasciculation near the edge of the explant may be the result of some other process rather than differential adhesion although it is not clear what that process may be.

Rutishauser & Edelman (1980) observed that the halo of outgrowth around dorsal root ganglia cultured in agar, on plastic and on collagen decreased sharply at NGF concentrations above 12 ng/ml. The inhibition of outgrowth was correlated with a thickening of neurite fascicles seen at NGF concentrations above 12 ng/ml. They proposed that an increase in fasciculation leads to an increase in the elastic tension of the fascicle without a compensatory increase in its adhesion to the substratum. The resulting imbalance inhibits neurite growth. In the present study neurites growing out from floating ganglia were highly fasciculated. At an NGF concentration of 50 ng/ml there was a sharp decrease in the amount of outgrowth from floating ganglia. Neurites growing on PLYS or a PORN and laminin coated substratum showed much less fasciculation.

Neurotrophic factor bioassay

Neurotrophic molecules such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) support the growth and survival of developing neurons and promote the outgrowth of their

neurites (Barde *et al* 1982; Levi-Montalcini 1987; Hohn *et al* 1990; Maisonpierre *et al* 1990; Thoenen 1991). Primary cultures of embryonic chick neural tissue are widely used for bioassay of known neurotrophic factors and in the search for novel factors. The quantitative effects of neurotrophic factors in regulating neurite outgrowth are most commonly measured after first attaching neural explants or dissociated cells to an adhesive substrate, or sometimes after embedding in a collagen gel (for example Letourneau 1975a,b; Davies 1989b; Ebendal 1989). These kinds of studies show that the amount and extent of fibre outgrowth also depends on the composition of the substrate. For example, the extracellular matrix protein laminin has an independent co-trophic effect brought about by a specific receptor-mediated interaction, and promotes the growth of neurites *in vitro* (Edgar *et al* 1984,1988; Bixby 1989).

The floating explant preparation was used to assay the effect of nerve growth factor on neurite outgrowth from E 7 DRG. The method is simple and effective. Time-consuming procedures involving preparation of glycoprotein matrices or coated substrata, and the adhesion of cells or explants to them are completely circumvented by this method. Floating explant preparations may therefore be especially well suited to investigation of neurotrophic factor activity in tissue extracts or conditioned media, especially when it is important to rule out possible co-trophic effects of adhesive substrata. In any case, the data shows that NGF-dependent neurite outgrowth is greater from floating DRG explants than from those adhered to polylysine, so the floating ganglia provides a more sensitive quantitative assay for neurotrophic factors than explants adhered to PLYS.

Over the last decade bioassays using primary neuronal culture have revealed selective neurotrophic activity from many sources including extracts from brain, neuroglia, and muscle (Barde *et al* 1982; Dohrmann *et al* 1986; Lumsden & Davies 1986; Hohn *et al* 1990; Maisonpierre *et al* 1990; Walicke, 1989). It may be interesting to study the effects of other growth factors of the neurotrophin family (such as BDNF, NT-3, NT-4, and NT-5; for review see Ebendal 1992) on neurite outgrowth from floating DRG preparations. The floating explant preparation is also potentially useful in conditioned media studies. Rather than collecting medium conditioned by cells or tissue explants and then adding that to dishes containing neural tissue in order to assay for neurite growth promoting activity, isolated ganglia could simply be positioned on the surface of the conditioned medium and neurotrophic activity quantified after an appropriate time.

It is interesting to note that explants of mammalian visual cortex or lateral geniculate nucleus also grow neurites along the surface of serum free culture medium (B. Lotto and D.J. Price, personal communication) but it remains to be seen whether floating explants prepared from other parts of the nervous system will grow neurites in the same way.

CHAPTER 4

INTRODUCTION

The cellular events which result in elongation of neurites and pathway selection by the advancing growth cone are dependent on dynamic yet highly organized cytoskeletal remodelling. Studies using drugs which disrupt microtubules and actin filaments have clearly shown both these cytoskeletal components to be involved. The function of microtubules is basically two fold: they form and maintain the structure of the cylindrical neurite; and as the substrate for fast axonal transport they facilitate delivery of organelles and newly synthesized membrane to the growth cone. The function of actin filaments is less clear: they are necessary for formation of filopodia and production of tension, and as the major component of the cortical cytoskeleton they are implicated in other cellular processes such as membrane flow, yet the fact that neurite elongation and even growth cone guidance can sometimes occur in the presence of cytochalasin B suggests that actin based subcellular events are not essential for neurite elongation.

A motile growth cone traversing through the environment of the embryo is constantly responding to extrinsic cue molecules which bind to cell surface receptors. When activated these receptors regulate actin dynamics in the growth cone. One of the primary functions of actin filaments is therefore to alter growth cone behaviour in response to extrinsic cue molecules. When neurites grow at an air-liquid interface there are no obvious extrinsic cue

molecules, and there is no substratum as such. In contrast neurites growing on a laminin substrate in culture are exposed to an extrinsic growth regulating molecule which affects adhesion and directly stimulates growth. Experiments were carried out using the drugs colcemid and nocodazole (which disrupt microtubules) and cytochalasin B (which disrupts actin filaments) to determine the role of these cytoskeletal components, and the contribution of " push " (microtubule based) and " pull " (actin based) mechanisms, towards neurite growth at an air-medium interface and adhered to laminin.

RESULTS

The effect of microtubule disorganizing drugs

Control cultures showed that there was considerably more outgrowth from ganglia adhered to a PORN and laminin coated substrate than from floating ganglia (Figs.4.1 and 4.5). Neurite elongation was inhibited when colcemid or nocodazole was added to the culture medium at the time of explantation. Increasing the concentration of colcemid or nocodazole in the culture medium systematically reduced the amount of neurite outgrowth from floating ganglia and from ganglia adhered to a laminin coated substratum. Inhibition of outgrowth from floating and adhered ganglia occurred over the same concentration range.

Fig.4.1

Neurite outgrowth after 24 h of culture in control medium containing 20 ng/ml NGF and DMSO. A, suspended at an air-culture medium interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.

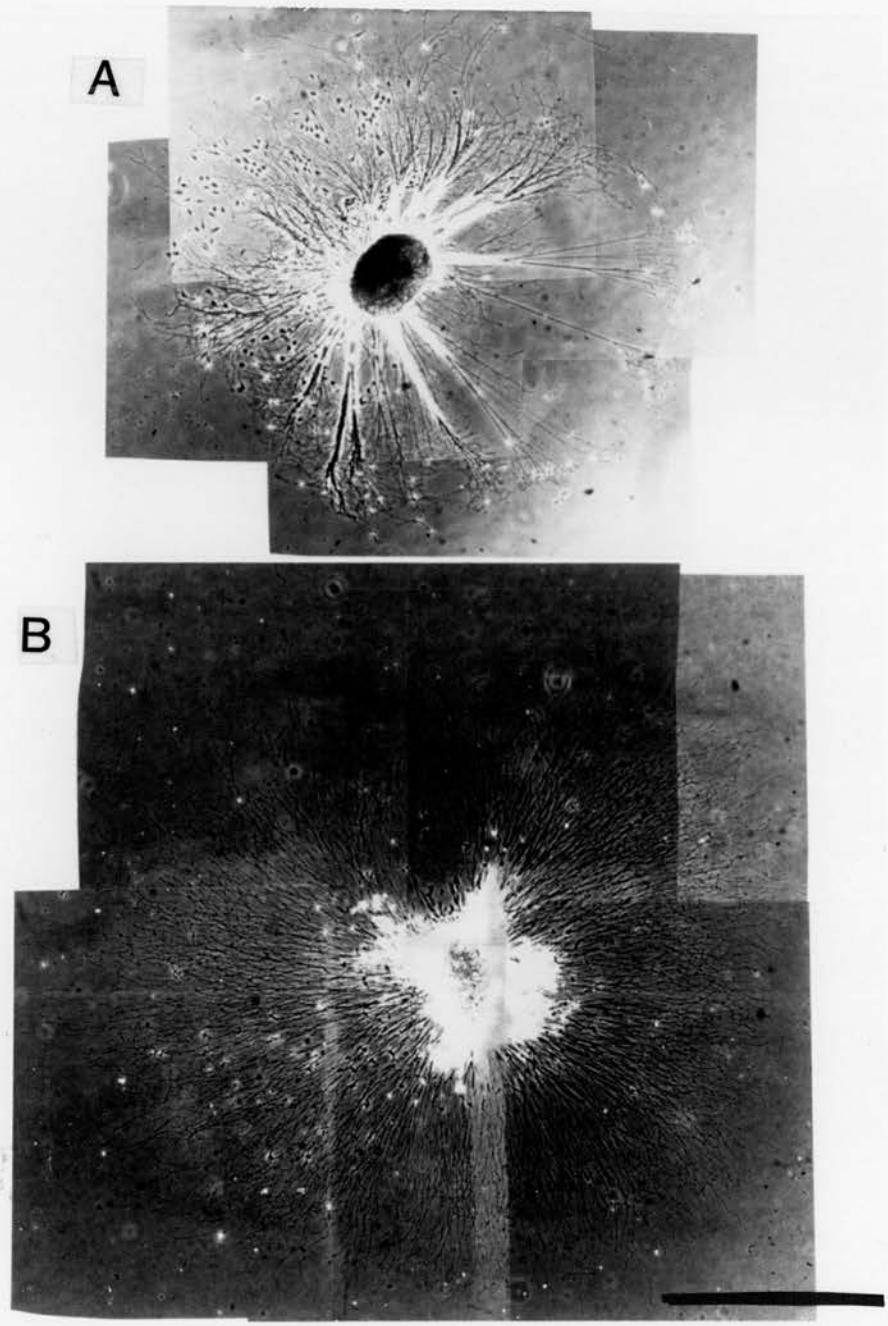

A
B

Fig.4.2

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.05 μg/ml nocodazole. A, suspended at the air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.

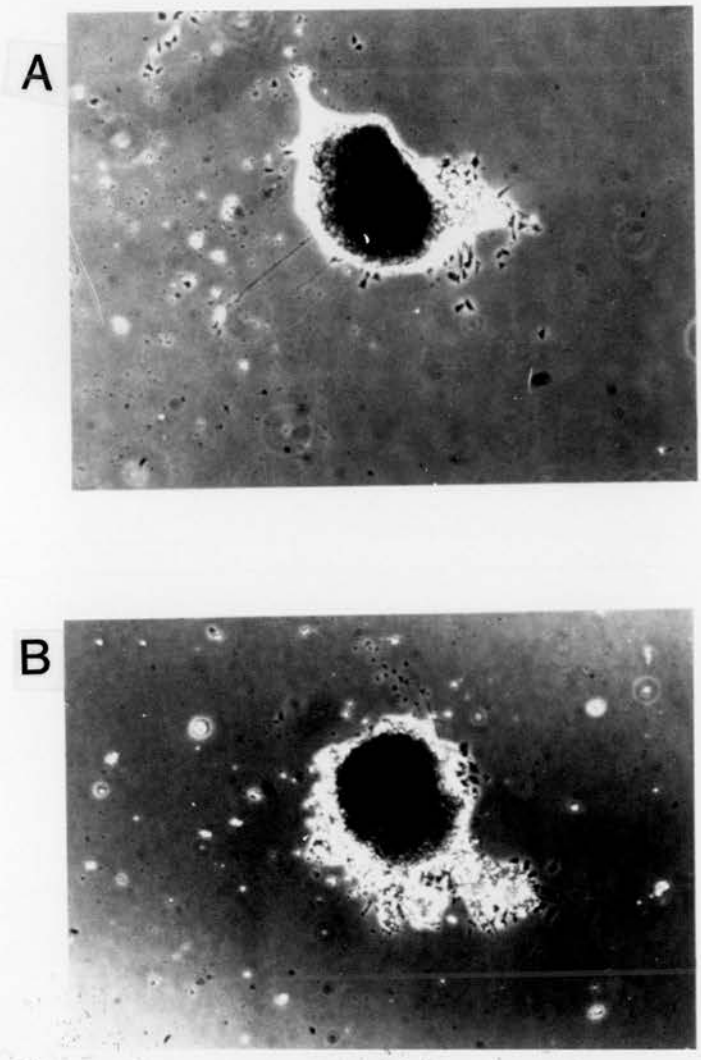
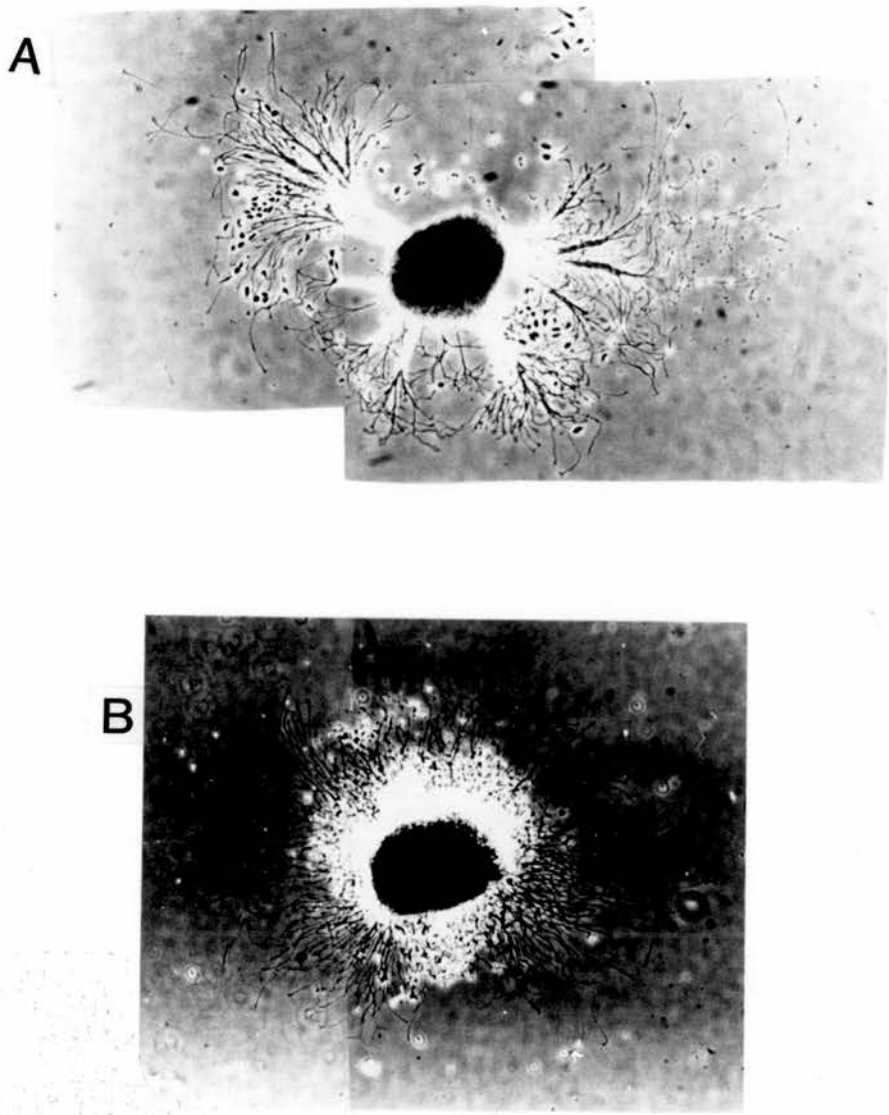

A
B

Fig.4.3

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.01 μg/ml nocodazole. A, suspended at the air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.


A
B

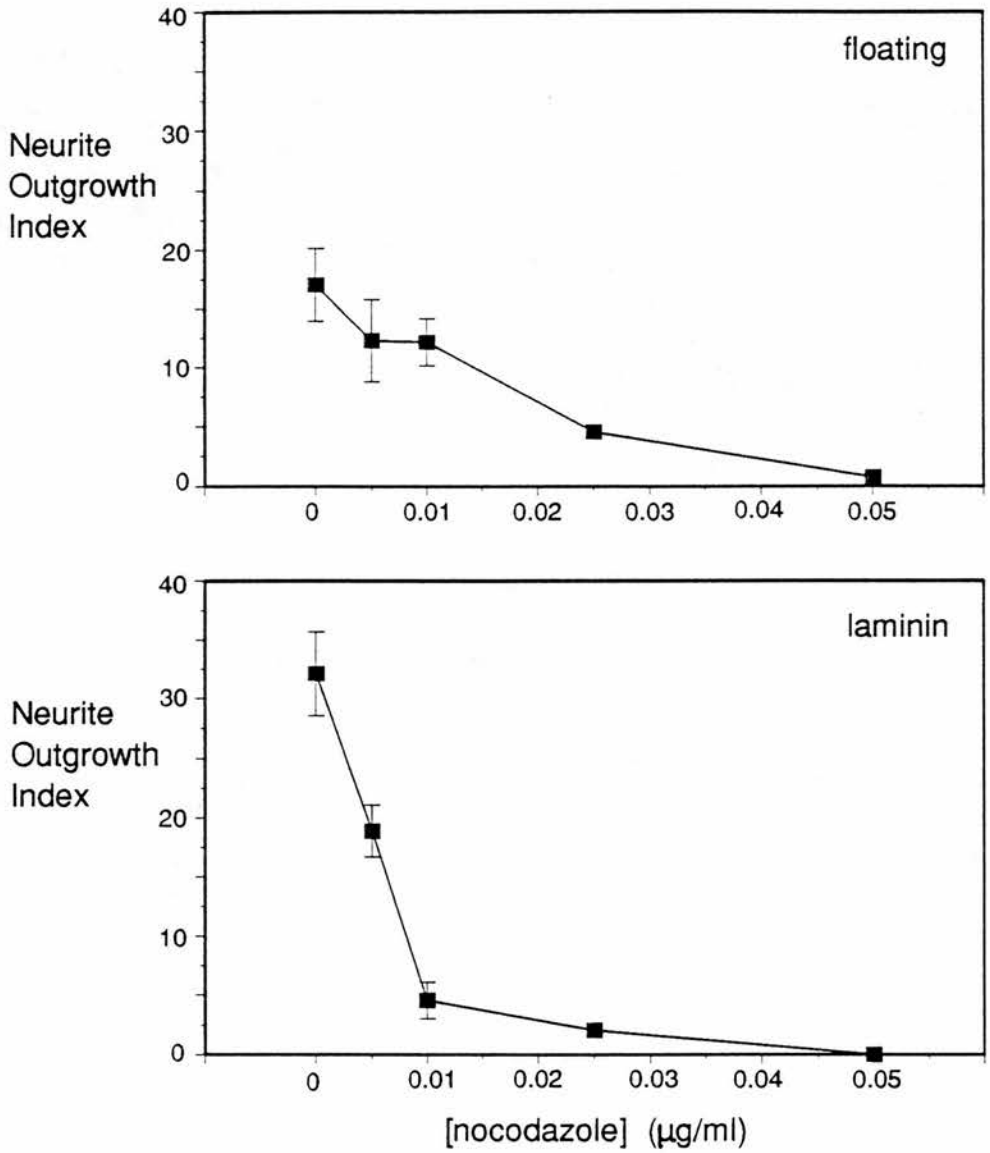


Fig.4.4

Neurite outgrowth after 24 h of culture in medium containing nocodazole and 20 ng/ml NGF. Each point shows the mean±S.D. obtained from between six and ten cultures.

Nocodazole was the more effective inhibitor, completely preventing neurite outgrowth from both floating and adhered DRG in medium containing 0.05 μg/ml (Fig.4.2). Growth of neurites adhered to laminin was more sensitive to nocodazole treatment than growth from floating ganglia (Fig.4.3). For example, in 0.01μg/ml nocodazole the neurite outgrowth index for adhered ganglia was reduced to less than a seventh of that in nocodazole free medium (from 32.15±3.58 to 4.55±1.55; Fig.4.4). In contrast the neurite outgrowth index for floating ganglia in 0.01 μg/ml nocodazole (17.01±2.54) was not statistically different from that in nocodazole-free medium (12.08±2.01; Wilcoxon unpaired test, P>0.05; Fig.4.4).

Colcemid was a less effective inhibitor of neurite outgrowth. Complete inhibition of neuronal elongation from floating and adhered ganglia occurred in medium containing 0.5 μg/ml, compared with 0.05 μg/ml nocodazole. The potency of nocodazole has been reported by other workers, for example Bamburg *et al* (1986). Growth on PORN and laminin coated substrata was again more sensitive than growth from floating DRG (Figs.4.6 and 4.7). In 0.05 μg/ml colcemid the outgrowth index for DRG on laminin (14.66±1.52) is about a half of the control value (27.02± 3.36; Fig.4.8) whereas the neurite outgrowth index for floating ganglia (10.80±2.20) was not significantly different from the control (10.31±1.22; Wilcoxon unpaired test, P>0.05; Fig.4.8).

Outgrowth seen when floating ganglia were cultured in low concentrations of nocodazole and colcemid appeared similar to that seen in the absence of the drugs. Neurites were highly fasciculated, outgrowth was commonly biased or patchy and large growth cones with many filopodia were clearly visible. However, migration of non-neuronal cells

Fig.4.5

Neurite outgrowth after 24 h of culture in control medium containing 20 ng/ml NGF. A, suspended at the surface of culture medium. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.

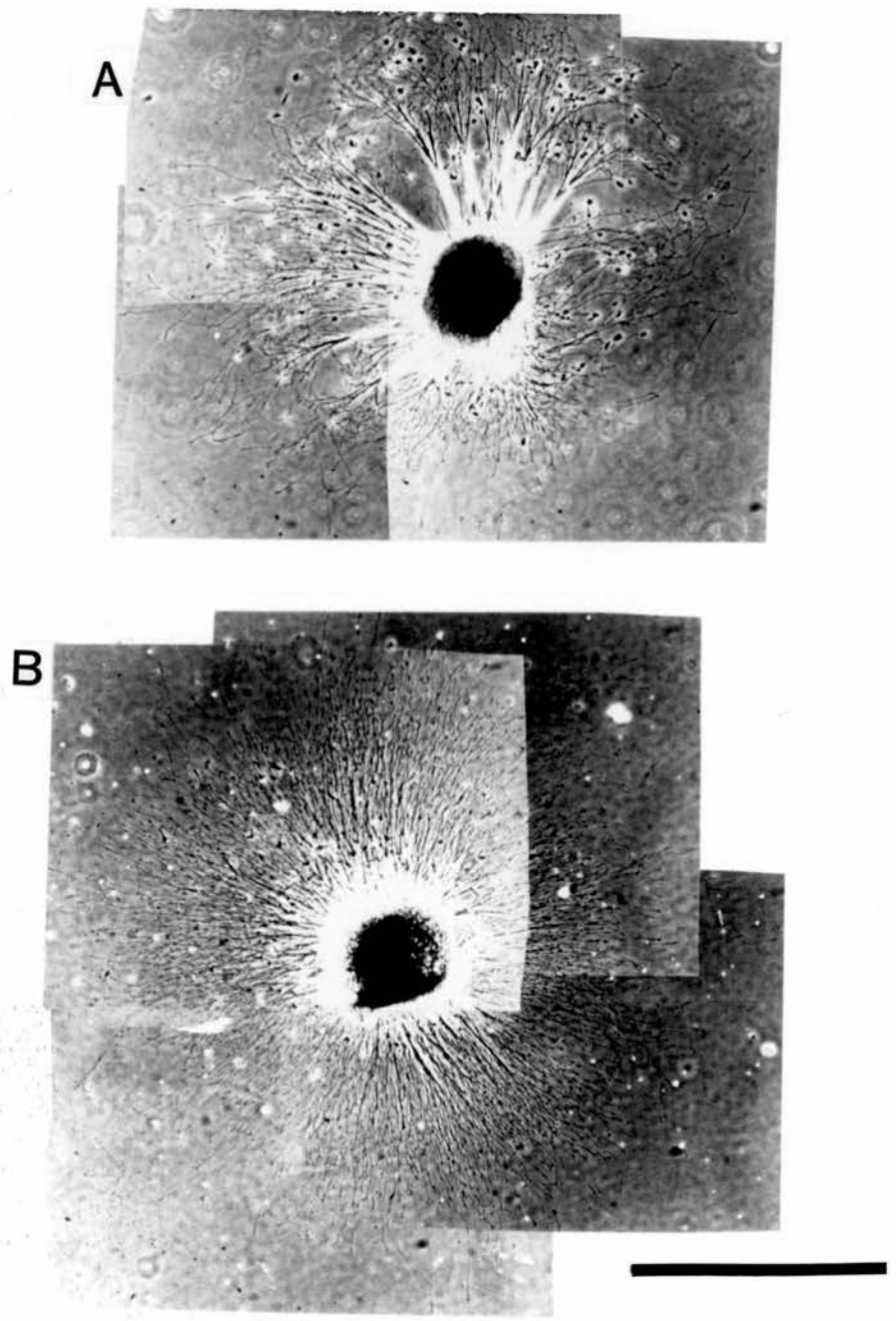

A
B

Fig.4.6

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.2 µg/ml colcemid (A,B) and 0.5 µg/ml colcemid (C,D). A and C, floating at the surface of culture medium. B and D, on a PORN and laminin coated substrate. Calibration bar, 1 mm.

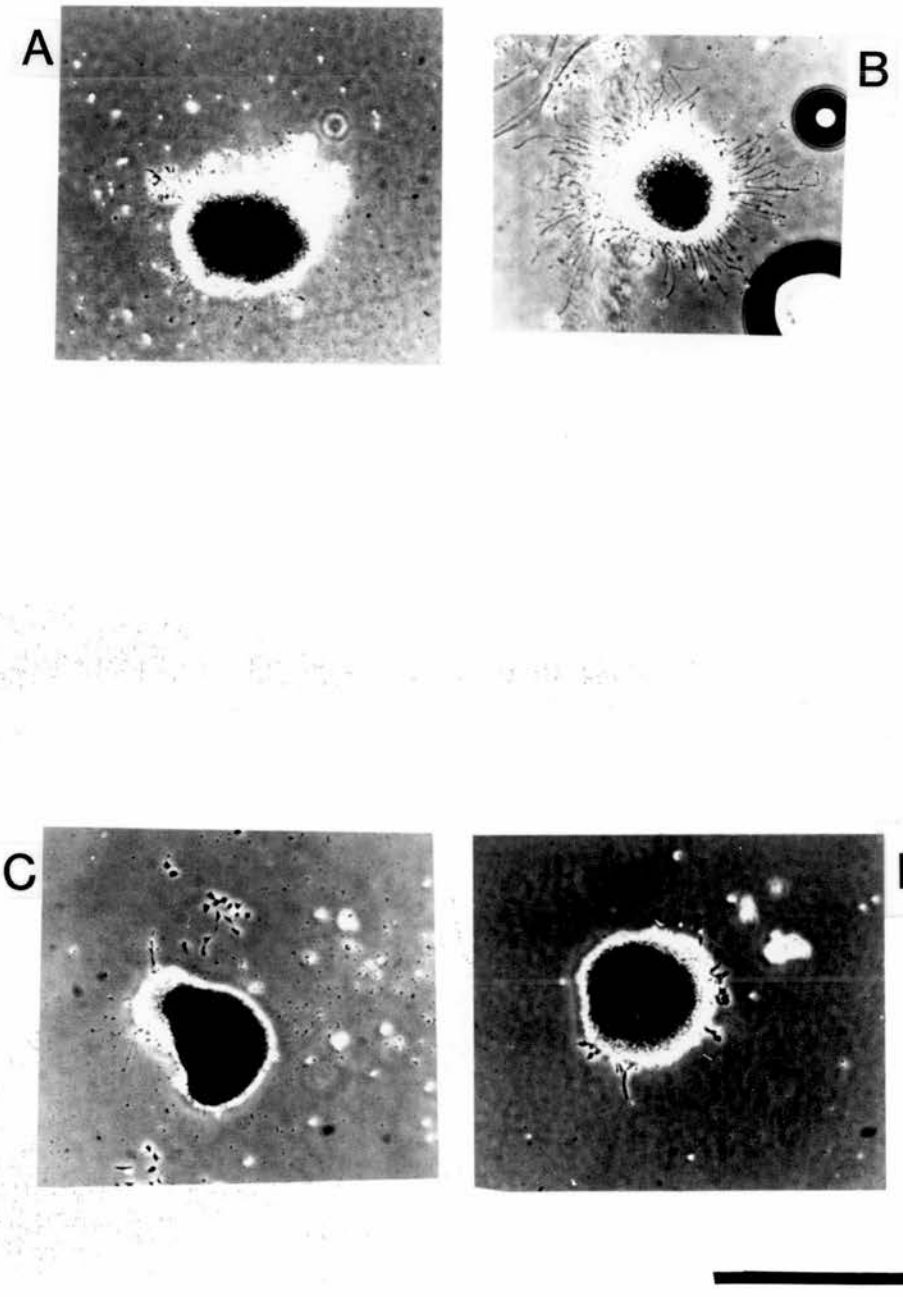
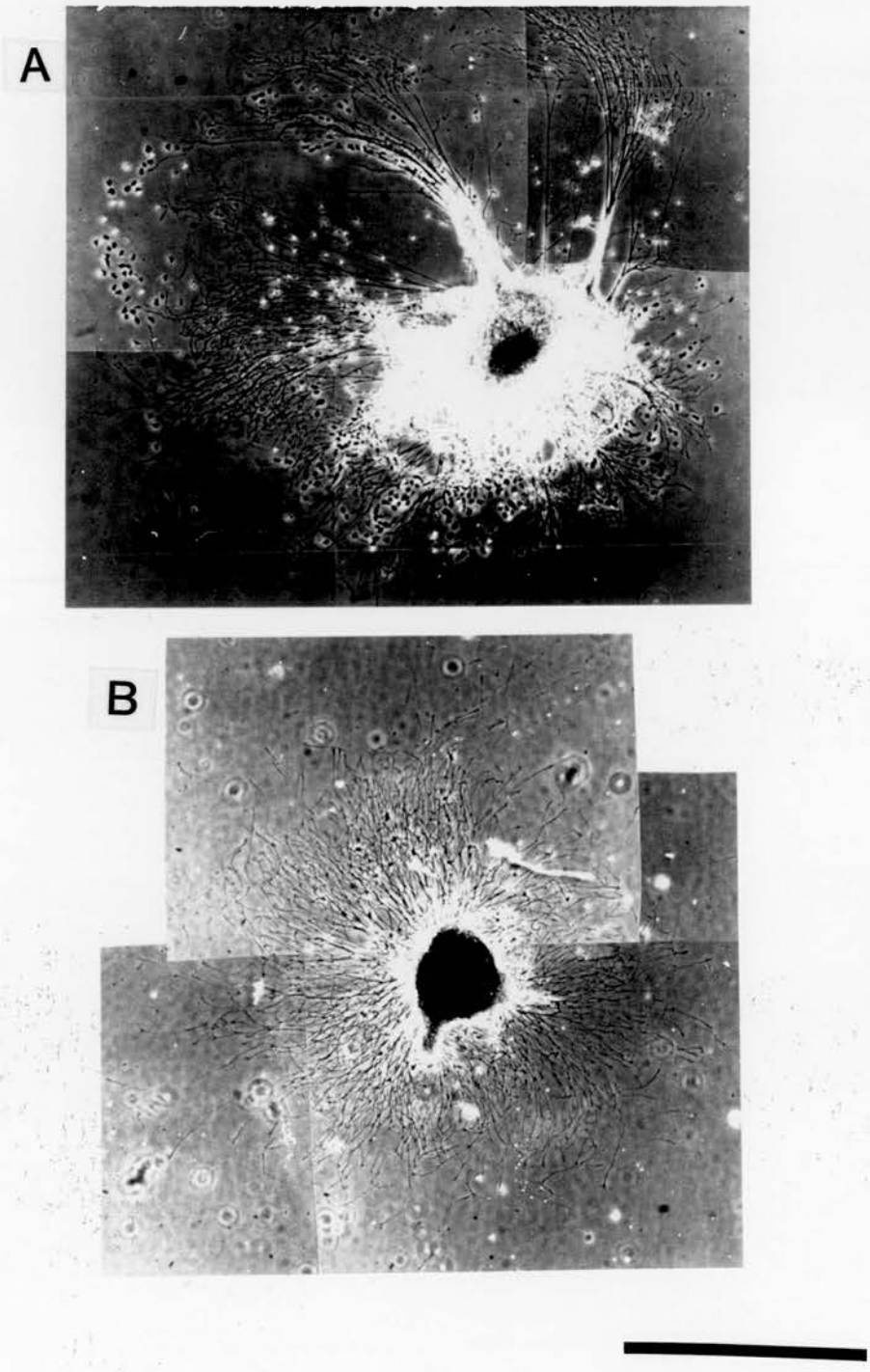

A
B
C
D

Fig.4.7

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.1 μg/ml colcemid. A, suspended at the air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.


A
B

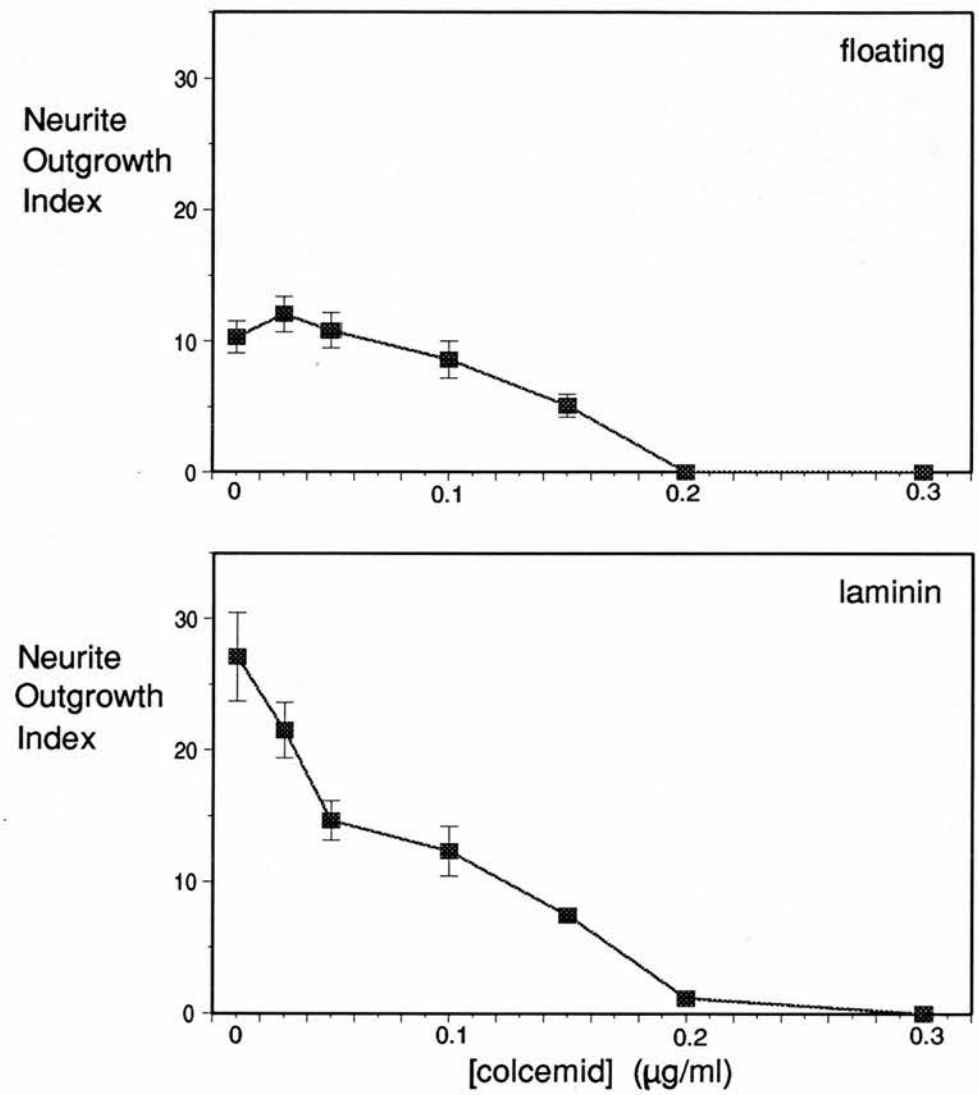


Fig.4.8

Neurite outgrowth after 24 h of culture in medium containing colcemid and 20 ng/ml NGF. Each point shows the mean±S.D. obtained from between six and ten cultures.

was completely inhibited (Figs.4.2 and 4.7)

The effect of actin filament disorganizing drugs

Neurites elongating from floating DRG and from ganglia adhered to laminin responded differently to cytochalasin B in the culture medium. Neurite outgrowth from floating ganglia decreased to approximately a third of its control value in medium containing 0.3 μg/ml cytochalasin B (from 19.99±1.56 to 7.02±0.76; Figs.4.9 and 4.10) before levelling off as the cytochalasin B concentration increased (Fig.4.11). Even in 0.7 μg/ml cytochalasin B, neurites continued to grow considerable distances from floating ganglia (Fig.4.12).

Outgrowth from ganglia adhered to PORN and laminin coated substrates fell to approximately half its control value in medium containing 0.1 μg/ml cytochalasin B (from 39.13±7.14 to 18.16±3; Fig.4.11). However, in contrast to neurite elongation from floating ganglia, neurite outgrowth on laminin continued to decrease as the concentration of cytochalasin B rose and was completely inhibited by 0.3 μg/ml (Figs.4.10 and 4.11).

The tips of neurites elongating from floating ganglia cultured in medium containing high concentrations of cytochalasin B were observed in order to assess growth cone morphology. Large expansive growth cones were generally absent, as would be expected in medium containing cytochalasin B, and the elongating neurites were generally tipped with short tapering processes. However, the overall pattern of outgrowth remained similar to that seen in the absence of the drug, with neurites commonly fasciculated and outgrowth frequently biased in direction.

Again, the migration of non-neuronal cells was completely inhibited.

Fig.4.9

Neurite outgrowth after 24 h of culture in control medium containing 20 ng/ml NGF and DMSO. A, suspended at the air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.

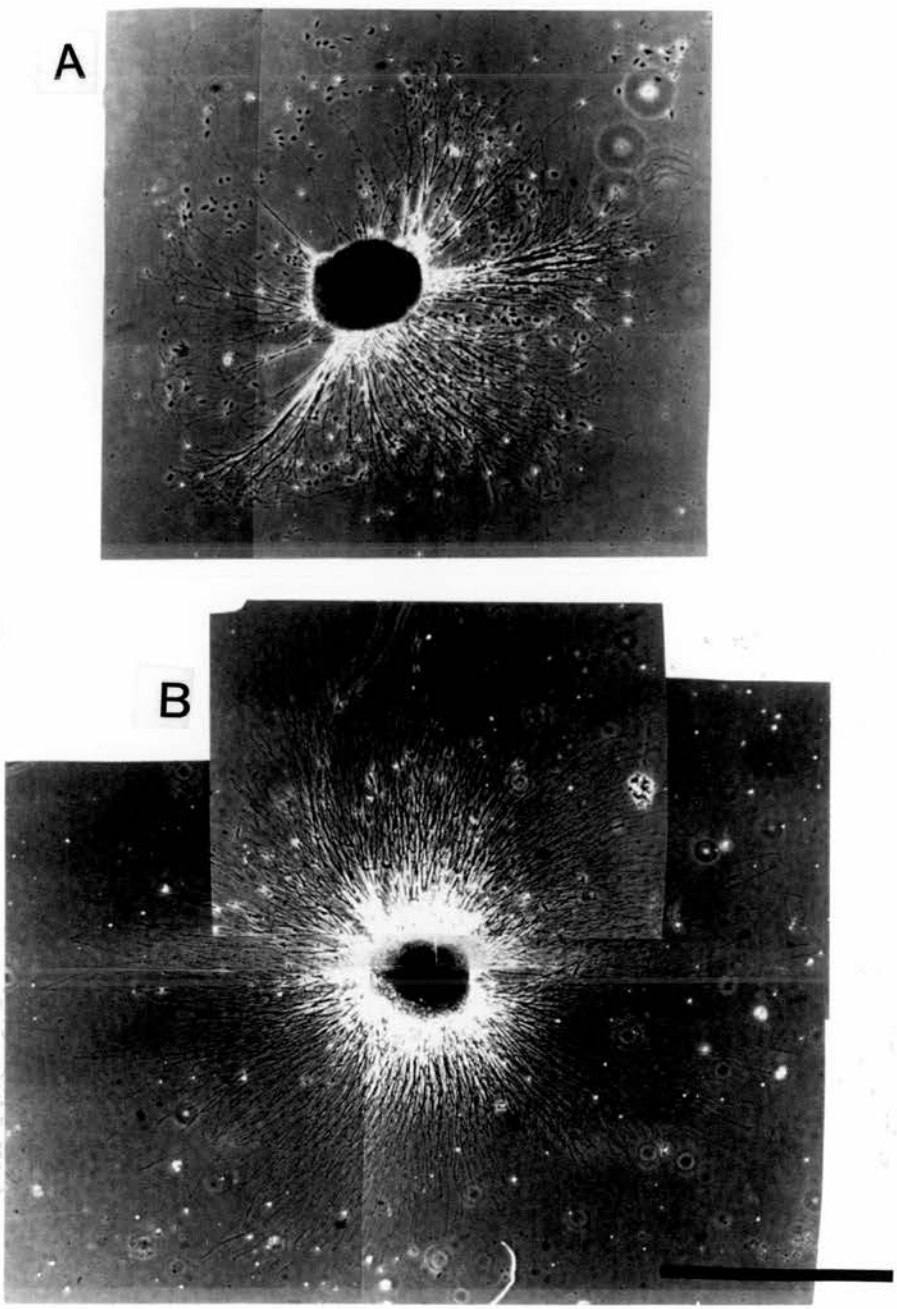
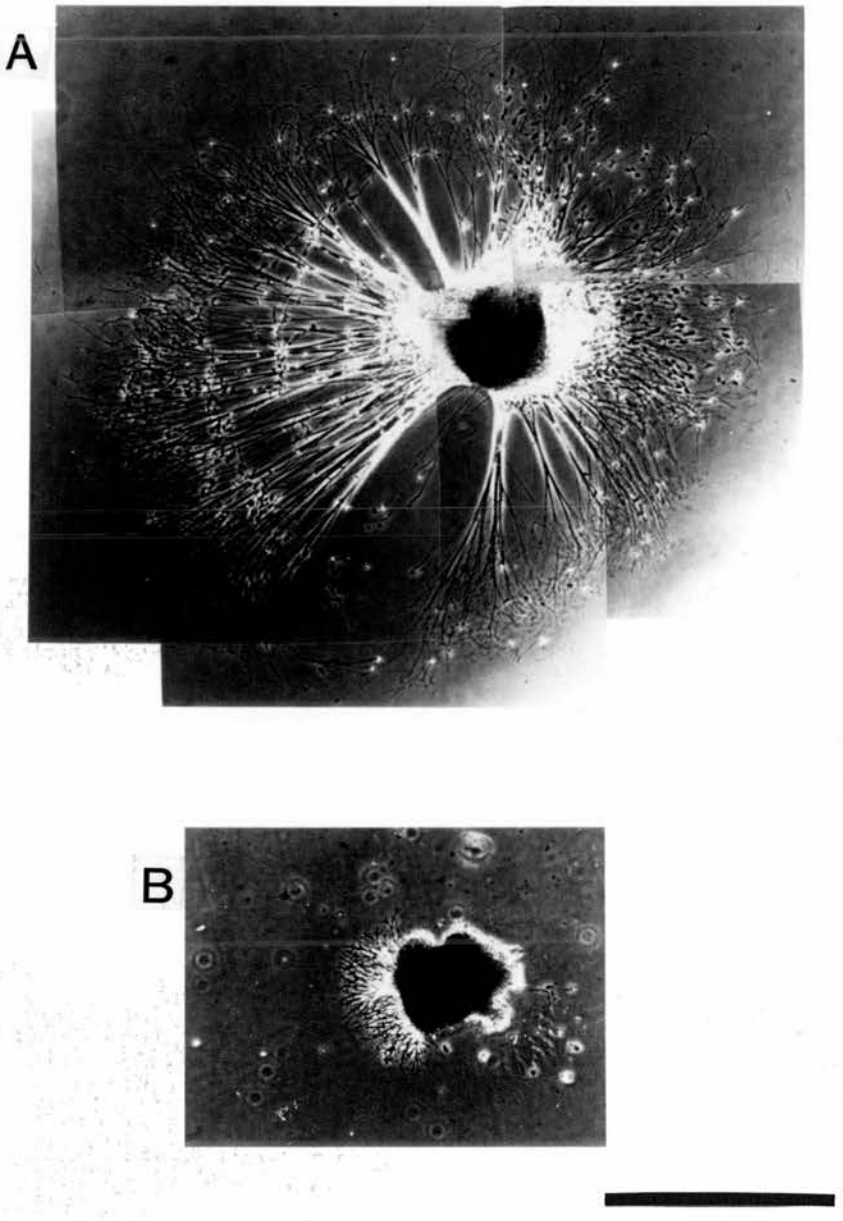

A
B

Fig.4.10

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.3 μg/ml cytochalasin B. A, suspended at the air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.


A
B

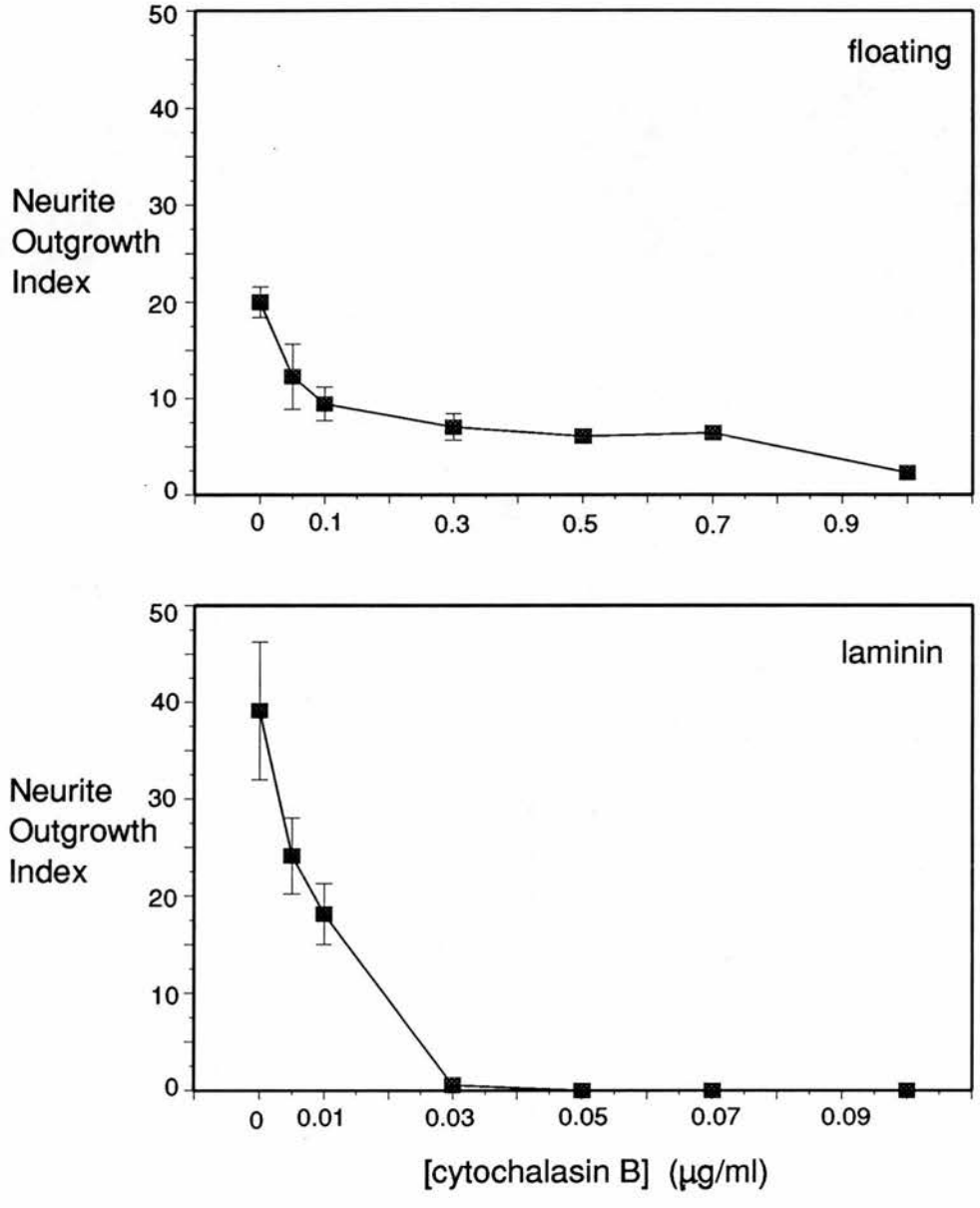
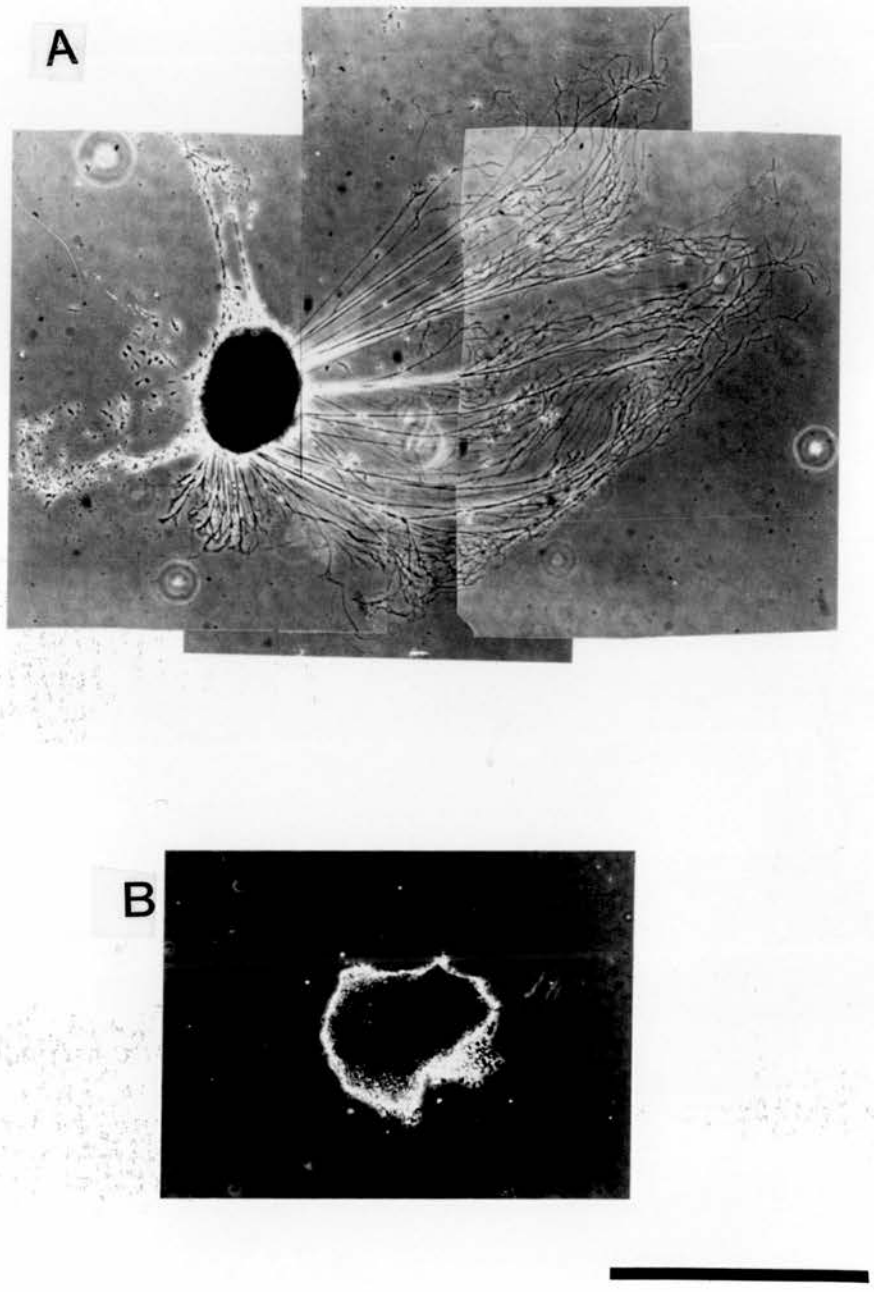


Fig.4.11

Neurite outgrowth after 24 h of culture in medium containing cytochalasin B and 20 ng/ml NGF. Each point shows the mean±S.D. from between six and ten cultures.

Fig.4.12

Neurite outgrowth after 24 h of culture in medium containing 20 ng/ml NGF and 0.7 μg/ml cytochalasin B. A, suspended at an air-liquid interface. B, on a PORN and laminin coated substrate. Calibration bar, 1 mm.


A
B

DISCUSSION

The role of microtubules and actin-filaments in neurite growth along the air-culture medium interface and over a laminin coated substrate was tested by measuring the amount of outgrowth which occurred in the presence of pharmacological agents that affect cytoskeletal components. Neurons in both culture systems are unable to extend neurites in the presence of colcemid and nocodazole, drugs affecting microtubules. The other important finding is that growth of neurites from floating ganglia occurs in the presence of cytochalasin B (CB), which affects actin-filaments, whereas neurite growth on laminin is ultimately inhibited.

The outgrowth of neurites along the underside of an air-liquid interface has not previously been described and the cellular mechanisms involved are unclear (see previous chapter). The adhesive interaction between the cell surface and fluid-interface is particularly obscure and the role of actin-based traction is difficult to determine. Neurite elongation on solid substrata generally depends on actin-containing extensions of growth cones adhering sufficiently strongly to external surfaces to allow tension development (Bray 1982, 1991). Filopodial extension and cellular traction are regulated by cytoskeletal activity. It is therefore of interest to determine the effects of drugs which disrupt cytoskeletal elements on neurite growth at a fluid-interface. This will provide information on the cytoskeletal basis of neurite elongation and may reveal something of the cell-substrate adhesive interactions.

Previous studies with neurons cultured *in vitro* have shown that neurite outgrowth is affected by microtubule-depolymerizing drugs, such as nocodazole, colchicine and the colchicine analogue colcemid, as well as by the actin-filament-disorganizing drugs, the cytochalasins (Yamada *et al* 1970, 1971; Daniels 1972; Marsh & Letourneau 1984; Bamburg *et al* 1986; Forscher & Smith 1988). In these studies, different effects were observed on different culture substrata. For example, Yamada *et al* (1970, 1971) and Marsh & Letourneau (1984) found that addition of CB to cultures of dissociated DRG neurons adhered to tissue culture plastic resulted in complete inhibition of neurite outgrowth or cessation of elongation from pre-existing neurites. However, neurons adhered to a polyornithine coated substrate can extend neurites for several hundred microns in the presence of CB (Marsh & Letourneau 1984). Dissociated *Aplysia* neurons adhered to a polylysine coated surface are also capable of extending neurites in the presence of CB (Forscher & Smith 1988). The effects of microtubule depolymerizing agents appears to be less dependent on the composition of the culture substrate. Colchicine and colcemid completely inhibit neurite elongation from dissociated DRG neurons on tissue culture plastic (Yamada *et al* 1970, 1971), collagen coated coverslips (Daniels 1972), and polyornithine coated surfaces (Bamburg *et al* 1986). Nocodazole prevents neurite outgrowth on polyornithine (Bamburg *et al* 1986). However, substrates coated with corneal-endothelial-cell-ECM support neurite elongation from PC12 cells in the presence of nocodazole, and therefore without microtubule assembly (Lamoureux *et al* 1990). Thus a neuron's ability to extend neurites in the presence of agents that compromise cytoskeletal integrity depends on the composition of the substrate.

Originally I thought that the effects of nocodazole, colcemid and CB on neurite outgrowth from floating ganglia could best be compared with their effects on neurite growth over polylysine or tissue culture plastic on which studies have already been made (Yamada *et al* 1970; Marsh & Letourneau 1984; Letourneau *et al* 1987; Forscher & Smith 1988). However, the results of this thesis show that DRG explants adhered to these substrata produce very little neurite outgrowth (see chapter 3) and the effects of these drugs would therefore be difficult to show. Consequently, the substrate chosen for comparison was PORN/laminin. In addition to providing a comparison, the effects of agents which disrupt cytoskeletal filaments have not previously been studied on laminin. Bray's theory (1991) that filopodial extension and tension development are only required to allow growing neurites to respond to extrinsic guidance cues can therefore be tested by observing the effects of CB on neurite growth over laminin, a putative guidance molecule *in vivo*.

The role of microtubules

In addition to the ability of nocodazole and colcemid to inhibit neurite outgrowth on tissue culture plastic, polyornithine and collagen the present results show that these drugs also inhibit neurite elongation at an air-liquid interface and on laminin. The primary target of these drugs is the assembly of microtubules (Margolis & Wilson 1977; Bergen & Borisy 1983; Zieve *et al* 1980). Thus neurite outgrowth at an air-liquid interface and on a PORN and laminin coated substratum depends on the assembly of microtubules, the major structural elements of the axon. Colcemid and nocodazole were added to cultures prior to the outgrowth of neurites. Consequently, initiation of axonal growth was inhibited in these cultures.

Little is known about the molecular mechanisms that regulate microtubule assembly and stabilization in growing neurites. Microtubules are formed by the GTP-dependent polymerization of tubulin monomers (Kirschner & Mitchison 1986). Polymerization usually starts from a nucleation site and occurs at both ends of the growing microtubule. Assembly is slow at the end anchored to the nucleation site (the minus end), but very rapid (4-10 μm/min; Schulze & Kirschner 1988) at the distal (plus) end. Nascent microtubules are highly unstable and are liable to collapse (Kirschner & Mitchison 1986). Stability is conferred by binding of microtubule associated proteins (MAP's) or covalent modification of tubulin by tyrosine or acetyl groups (Matus 1988; Cambrey-Deakin & Burgoyne 1987). Axonal microtubules are oriented with their fast growing (plus) ends distal to the cell body (Heidemann *et al* 1981), and the principle assembly site is at the base of the growth cone (Bamburg *et al* 1986).

Microtubules are the most significant structural component of the axon and it is difficult to see how neurite elongation could proceed without microtubule assembly. However, Lamoureux *et al* (1990) found that addition of nocodazole prior to neurite outgrowth from PC12 cells did not inhibit neurite elongation on ECM material even at concentrations as high as 0.5 μg/ml. They suggest that ECM stimulates the actin-based activity of the growth cone thereby increasing tension development to such a degree that a neurite is " pulled out ". Neurites formed under these conditions do not contain microtubules. Laminin has been found to stimulate growth cone-like behaviour in the neuronal cell line NG108-15 (Smalheiser 1989), and may be the active component of the corneal-endothelial-cell-ECM used by Lamoureux *et al*. However, the present finding that DRG neurite outgrowth is completely inhibited on laminin by 0.05 μg/ml nocodazole

suggests that either laminin is not the active ECM component or that significant differences exist between DRG neurons and PC12 cells in the cellular mechanism of neurite growth.

In summary: the finding that colcemid and nocodazole completely inhibit neurite elongation from floating DRG and from DRG adhered to laminin coated substrata suggests, firstly,that extension of neurites from floating ganglia, as well as those adhered to laminin, requires formation of an axonal cylinder supported by a core of stable microtubules. This result supports the proposal that neurite growth depends on a " push " mechanism, but does not preclude the possibility that an actin based, or " pull ", mechanism may also operate. Secondly, growth cone adhesion at a fluid-interface or on laminin does not increase actin-based activity and tension development sufficient to promote neurite growth in the absence of microtubule assembly (see Lamoureux *et al* 1990).

The role of actin-filaments

Like microtubules, actin filaments are also polar. ATP-dependent polymerization occurs preferentially at the barbed end (Mitchison & Kirschner 1988; Pollard & Cooper 1986). Cytochalasins inhibit actin polymerization by capping the barbed ends (MacLean-Fletcher & Pollard 1980) or severing filaments (Hartwig & Stossel 1979).

The present experiments using cytochalasin B were undertaken to test the role of filopodial activity and tension development, and consequently the role of an actin-based " pull " mechanism, in neurite outgrowth across an air-liquid interface and on a laminin substrate. The results obtained show

that neurite elongation at a fluid interface occurs at high cytochalasin B concentrations, up to 1.0 μg/ml, whereas growth on laminin is completely inhibited by 0.5 μg/ml. Thus actin-based traction does not appear to be essential for neurite elongation at an air-liquid interface, but is required for neurite growth on laminin.

In vitro, neurites with growth cones deprived of filopodia by cytochalasin treatment are unable to elongate on tissue culture plastic (Yamada *et al* 1970; Marsh & Letourneau 1984), but do elongate on polyornithine or polylysine coated substrates (Letourneau *et al* 1987; Marsh & Letourneau 1984; Forscher & Smith 1988). This difference reflects the low adhesivity of tissue culture plastic, compared with highly adhesive polypeptides (Marsh & Letourneau 1984; Bray 1991). The failure of neurites lacking filopodia to grow on tissue culture plastic suggests that one of the principle functions of filopodia is to adhere to the substratum. On " sticky " substrata, such as polylysine, adhesion between the neurite and substratum is strong enough to allow elongation even in the absence of filopodia: adhesion being the key to successful growth. Gundersen (1988) used interference reflection microscopy (IRM) to study growth cone-substrate contacts.This technique is based on optical interference patterns generated by light reflected from the substrate and the membrane next to that substrate, and can be used to measure the closeness of association between the cell surface and underlying substrate. He showed that on a variety of surfaces, closest association occurs between the substrate and growth cone filopodia, suggesting that these are the sites of strongest adhesion. Letourneau (1978b) used IRM to show that growth cone-substrate contact is significantly closer on polylysine coated as opposed to untreated surfaces.

The ability of neurites lacking filopodia to elongate *in vitro* is believed to result from a strong adhesion between the neurite and its substratum. The results of this thesis show that outgrowth of neurites from floating DRG occurs in the presence of cytochalasin B and the absence of filopodia. This suggests a strong adhesive interaction between the air-liquid interface and the neuronal cell surface. Yet Bray (1991) states that growth cones on highly adhesive surfaces coated with polylysine are " aberrant in form and function " and are often not seen. In contrast, the present results show growth cones at an air-liquid interface are generally well spread and normal in appearence. Bentley & Toroian-Raymond (1986) studied the growth of grasshopper pioneer neurons *in vivo* deprived of filopodia by cytochalasin treatment. They found that axons were able to grow, albeit with irregular, wandering trajectories, and suggested this was due to the high adhesivity of the substrate. In the absence of cytochalasin, pioneer neuron growth cones were morphologically normal, well spread with many filopodia, despite being on a highly adhesive surface. Thus, growth of neurites tipped with morphologically normal growth cones can sometimes occur across highly adhesive substrata *in vivo*. The present results suggest this may be occurring at an air-liquid interface.

It was also found in the present study that in the presence of less than 0.1 μg/ml cytochalasin B, neurite outgrowth from floating ganglia appeared to be inhibited in a concentration dependent manner. However, at concentrations above this and as high as 1.0 μg/ml no further inhibition of growth occurred. This suggests that some form of actin-mediated process, possibly traction, contributes to neurite elongation but is not an essential requirement of the growth mechanism. Tension development and cellular traction are a consequence of cell-substrate adhesion strong enough to

resist being pulled against, and an immovable substrate against which to pull (Bray 1982; Singer & Kupfer 1986; Heidemann *et al* 1990). The viscoelastic monolayer present at an air-liquid interface may facilitate strong adhesion but it is unclear whether this substrate could be pulled against. If such a substrate could not support traction then neurite elongation would be entirely due to a protrusive mechanism based on microtubule assembly. However, the finding that neurite elongation is sensitive to cytochalasin B suggests that an actin-based process is contributing to neurite growth at an air-liquid interface, although the nature of this process is unknown.

The present finding that neurites are unable to elongate on laminin in the presence of cytochalasin B suggests laminin is a substrate of low adhesivity. Yet when sensory neurites are grown *in vitro* on substrata patterned with laminin, fibronectin and PLYS, laminin is the preferred substrate (Hammarback *et al* 1985, 1988; Gundersen 1987). Hammarback *et al* (1988) suggested that the preferential growth of sensory neurites on laminin reflected increased growth cone-substratum adhesion. However, Gundersen (1987) directly measured sensory growth cone adhesion to laminin, fibronectin and PLYS coated substrata and found that growth cone-substrate adherence on laminin (15.4 ± 0.5 lb/in^2) was significantly less than on PLYS (26.0 ± 0.3 lb/in^2) or fibronectin (19.8 ± 0.5 lb/in^2). Gundersen suggested that enhanced neurite elongation and substrate preference on laminin may be the result of cytoskeletal stabilization or activation of second messengers rather than increased adhesion. There is evidence to support this. Binding of laminin to calf adrenal chromaffin cells was shown by Acheson *et al* (1986) to increase the level and activity of tyrosine hydroxylase, and Bixby (1989) demonstrated that stimulation of

neurite outgrowth on laminin involves activation of protein kinase C via integrin receptors. My data suggest that laminin is not a highly adhesive substrate for neuronal growth cones and thus support the theory that stimulation of neurite outgrowth on laminin occurs via a neurotrophic rather than an adhesive mechanism.

Bray's proposal (1991) that filopodial activity may only be necessary to allow growth cones to respond to environmental guidance cues is also supported by the present finding that outgrowth on laminin, a putative guidance molecule *in vivo*, is completely inhibited in the presence of cytochalasin B.

In summary: the results presented in this thesis show that cytochalasin B completely inhibits neurite elongation from ganglia adhered to laminin but not from floating ganglia. This suggests that laminin is a poorly adhesive substrate and neurite extension on laminin requires filopodial activity and an actin-based " pull " mechanism. In contrast, an air-culture medium interface appears to be highly adhesive. Whether a fluid interface will permit tension development remains unclear. If not, neurite elongation must primarily result from protrusion of microtubules and other cellular components, the " push " mechanism.

CHAPTER 5

INTRODUCTION

During the development of sensory innervation, segmental differences arise in the rates of axonal growth from DRG located at different axial levels *in vivo*. Axons innervating developing limb buds grow faster than those which innervate non limb regions. It is not known how these differential growth rates are controlled. There are two major ways in which cellular differentiation may be controlled: intrinsic determination; or environmental regulation. The question addressed in this chapter is whether segmental differences in outgrowth seen *in vivo* are determined by intrinsic properties of the neurons. Isolating cells or tissues in culture generally removes environmental cues, so any differences in axonal growth rates seen *in vitro* would therefore be the result of intrinsic determination. However, if the culture substrate is composed of extracellular matrix molecules (laminin and fibronectin are used routinely in neuronal cell culture), membrane fragments or neurotrophic factors then it is possible that these may provide some growth regulating signals perhaps similar to those present *in vivo*. For example, laminin directly stimulates neurite growth (Bixby 1989). Molecules such as polylysine and polyornithine (which have no physiological role as neurotrophic factors *in vivo*) are unlikely to provide cues for differential axonal growth rates, but neurite outgrowth is far less extensive on these polypeptide substrates than on laminin or fibronectin (see chapter 3). Also Bray (1991) states that growth cones on sticky, polypeptide surfaces are " aberrant in form and

behaviour " and are often not seen at all.

Problems which may arise from culture on these substrates are avoided using the floating ganglion preparation. Outgrowth of neurites from floating dorsal root ganglia is greater than on polylysine (see chapter 3) so the floating ganglia provide a more sensitive method for determining whether segmental differences in axonal growth rate are intrinsically determined. Neurites growing from floating ganglia also possess expansive and highly active growth cones, morphologically similar to those seen *in vivo* (Harris *et al* 1987).

Experiments were carried out using dorsal root ganglia isolated from embryos at developmental stages 25, 28, 30 and 32 (corresponding approximately to days 4.5, 5.5, 6.5 and 8 of incubation). At stage 25 axons first begin to leave the ganglia and invade the proximal regions of the limb; by stage 32 axons have reached the tips of both fore and hind limbs, the patterning of nerves in the limbs has been established and target innervation is underway. Ganglia were taken from brachial, thoracic and lumbosacral segmental levels.

RESULTS

Neurite outgrowth from stage 25 to stage 30 ganglia in culture

Brachial, thoracic and lumbosacral dorsal root ganglia isolated from stage 25, 28 and 30 embryos were cultured for 24 h at the air-medium interface. No segmental differences in the amount of neurite outgrowth were

Fig.5.1

Effect of segmental level on neurite outgrowth from stage 25 dorsal root ganglia after 24 h of culture floating at the surface of serum-free culture medium. B, brachial; T, thoracic; L, lumbosacral. Calibration bar, 1 mm.

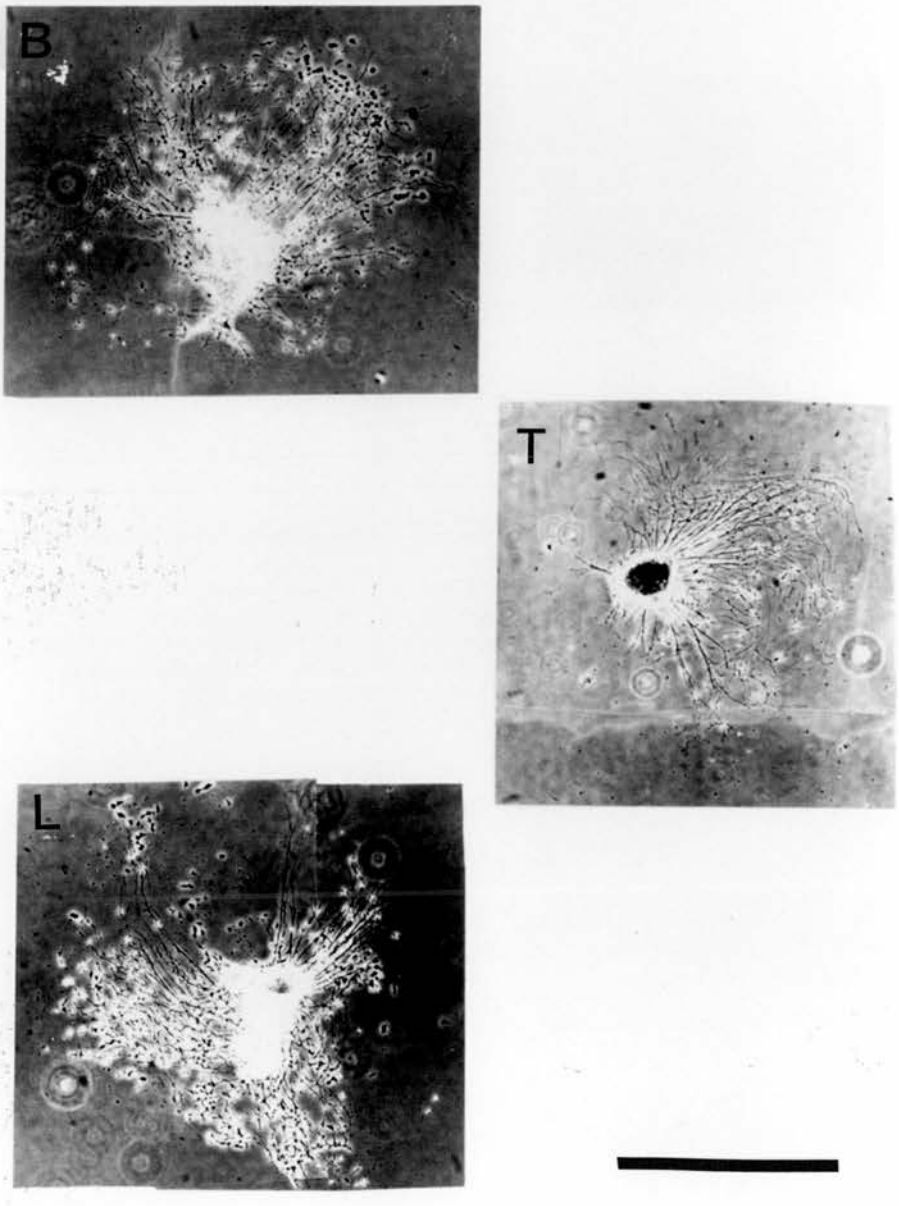

B
T
L

Fig.5.2

Effect of segmental level on neurite outgrowth from stage 28 dorsal root ganglia after 24 h of culture floating at the surface of serum-free culture medium. B, brachial; T, thoracic; L, lumbosacral. Calibration bar, 1 mm.

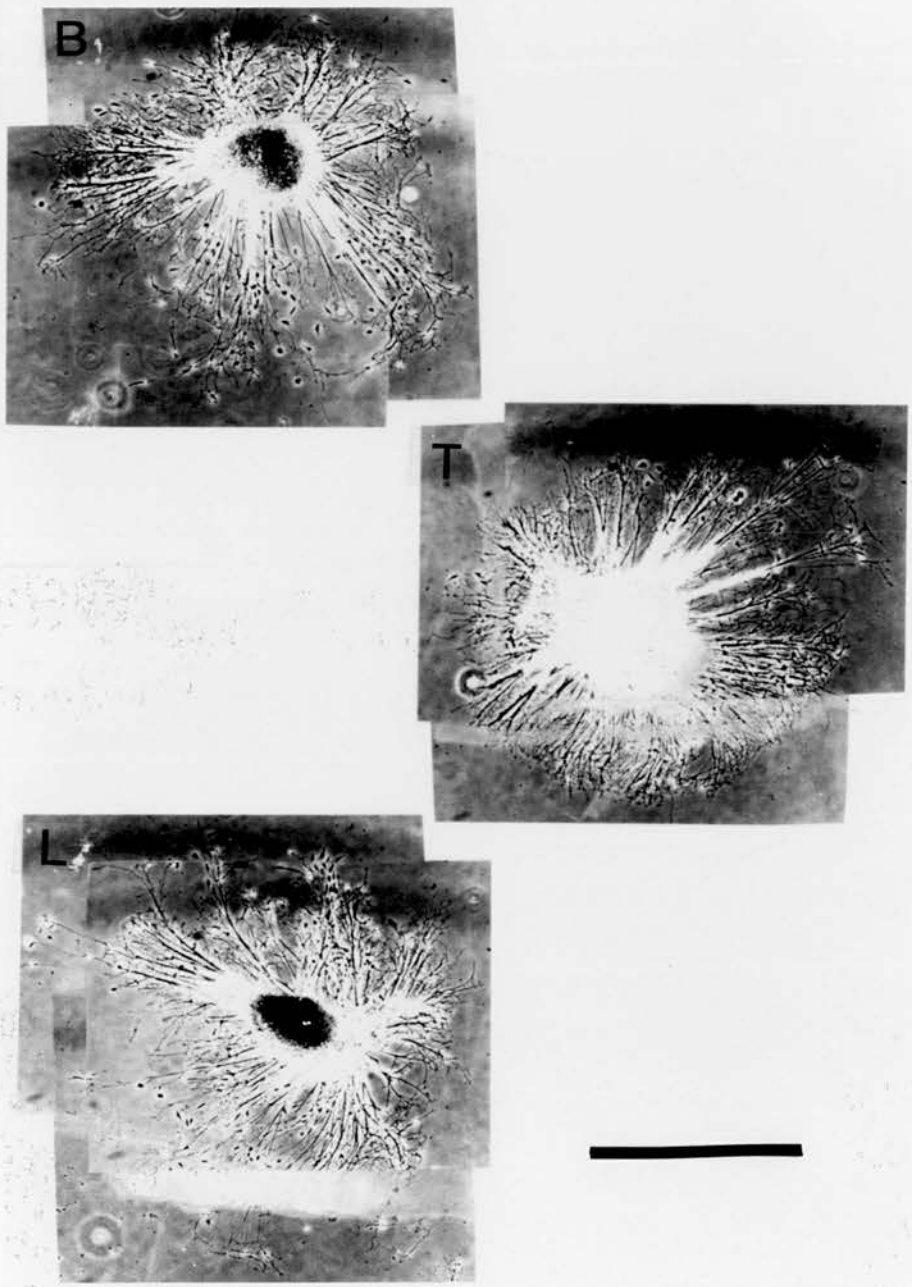

B
T
L

Fig.5.3

Effect of segmental level on neurite outgrowth from stage 30 dorsal root ganglia after 24 h of culture floating at the surface of serum-free culture medium. B, brachial; T, thoracic; L, lumbosacral. Calibration bar, 1 mm.

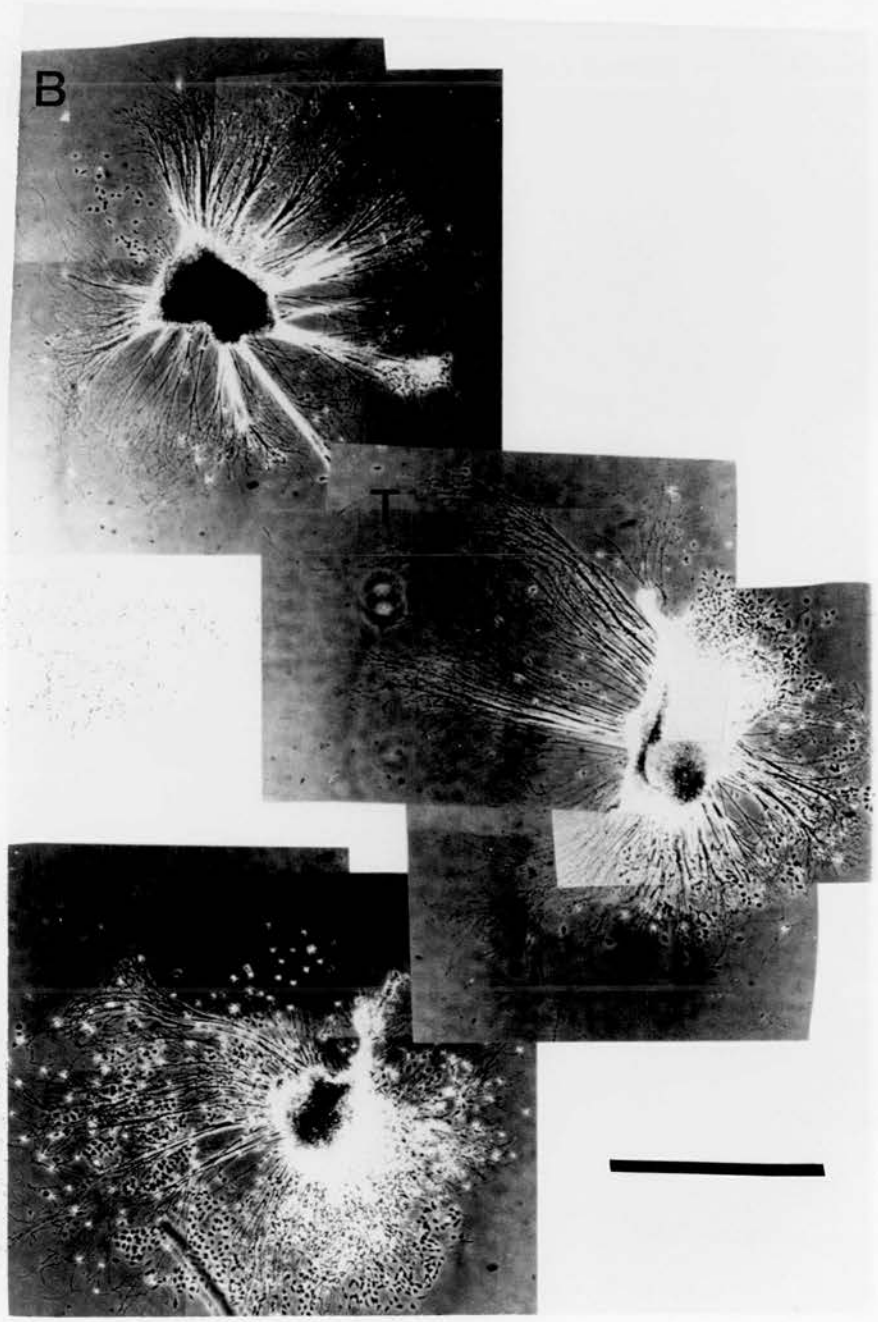
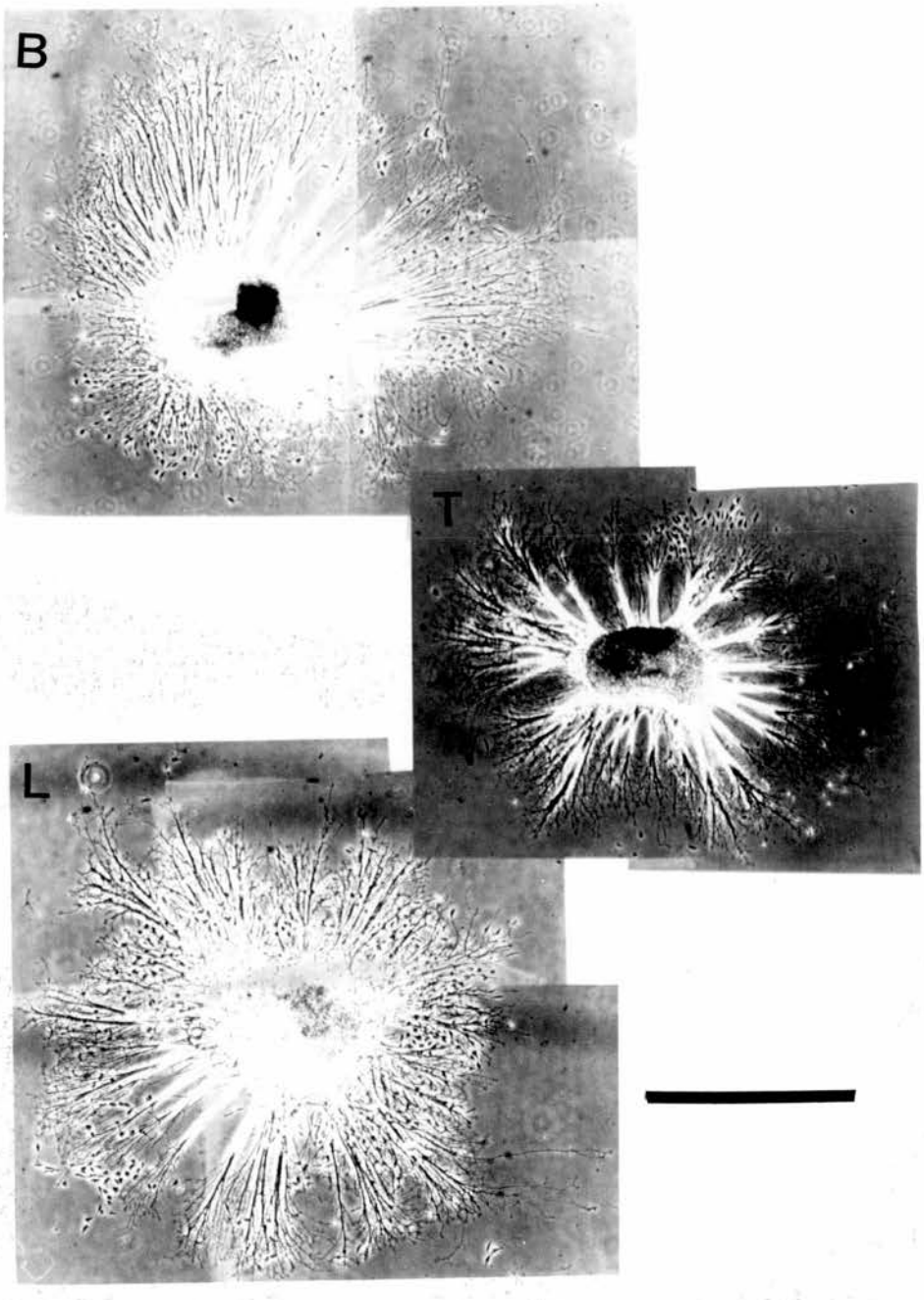

B
T
8

Fig.5.4

Effect of segmental level on neurite outgrowth from stage 32 dorsal root ganglia after 24 h of culture floating at the surface of serum-free culture medium. B, brachial; T, thoracic; L, lumbosacral. Calibration bar, 1 mm.


B
T
L

apparent at stage 25 (Fig.5.1), stage 28 (Fig.5.2) or stage 30 (Fig.5.3). In addition, no differences could be detected in the neurite outgrowth index for ganglia isolated from different segmental levels (Figs.5.5 and 5.6). No differences were seen in the appearance of ganglia from different segmental origins: neurites were always highly fasciculated and the patterns of outgrowth variable.

Neurite outgrowth from stage 32 ganglia in culture

Dorsal root ganglia isolated from different segmental levels of stage 32 embryos were cultured for 24 h floating at the surface of culture medium (Fig.5.4). In cultures containing thoracic ganglia the neurite outgrowth index was less than in cultures containing brachial or lumbosacral ganglia (Fig.5.6). The difference was statistically significant between thoracic and brachial ganglia and between thoracic and lumbosacral ganglia (Wilcoxon unpaired test, $P<0.05$ in each case).

The amount of neurite outgrowth from floating ganglia of different embryonic age

Ganglia removed from successively older embryos grew longer neurites *in vitro*. The neurite outgrowth index for DRG isolated from the brachial segmental level doubles in the 24 hr between stage 25 and stage 28. There is a small but significant increase between stage 28 and 30, and a further dramatic increase between stage 30 and 32 (Fig.5.7). Ganglia isolated from lumbosacral segmental levels show a similar pattern of increasing amounts of neurite outgrowth *in vitro* (Fig.5.8). However, ganglia innervating thoracic targets show a different pattern of growth.

Between stage 25 and 30 the pattern of increasing neurite outgrowth index for these ganglia is similar to that for brachial and lumbosacral ganglia. The dramatic rise in the amount of growth between stage 30 and 32 which is seen in limb innervating ganglia is, however, not found in thoracic ganglia (Fig.5.9).

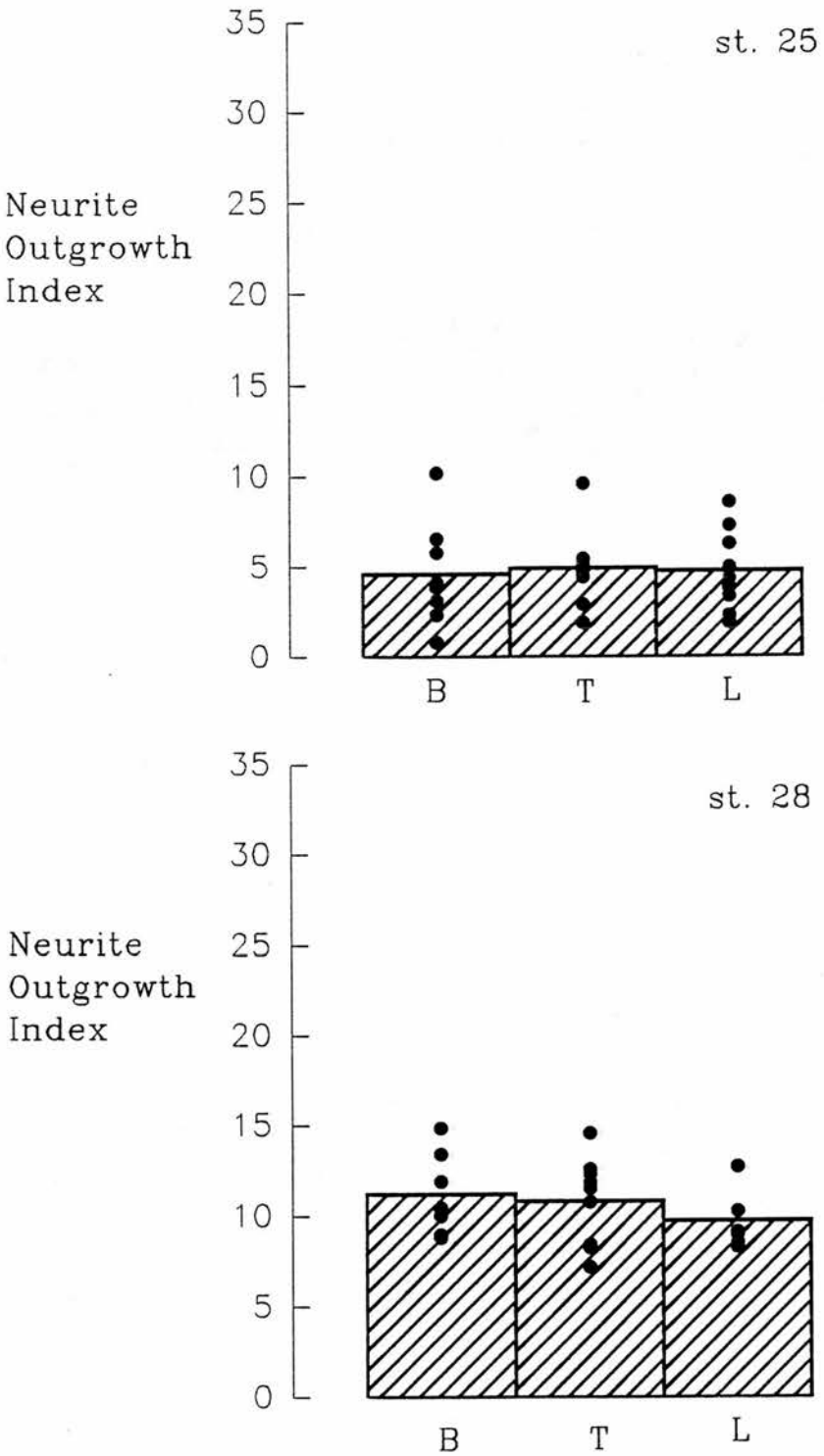


Fig.5.5

Neurite outgrowth after 24 h of culture from dorsal root ganglia isolated from stage 25 and 28 embryos. Bars height represents mean of explant scores for brachial (B), thoracic (T) and lumbosacral (L) ganglia, and data points are also shown.

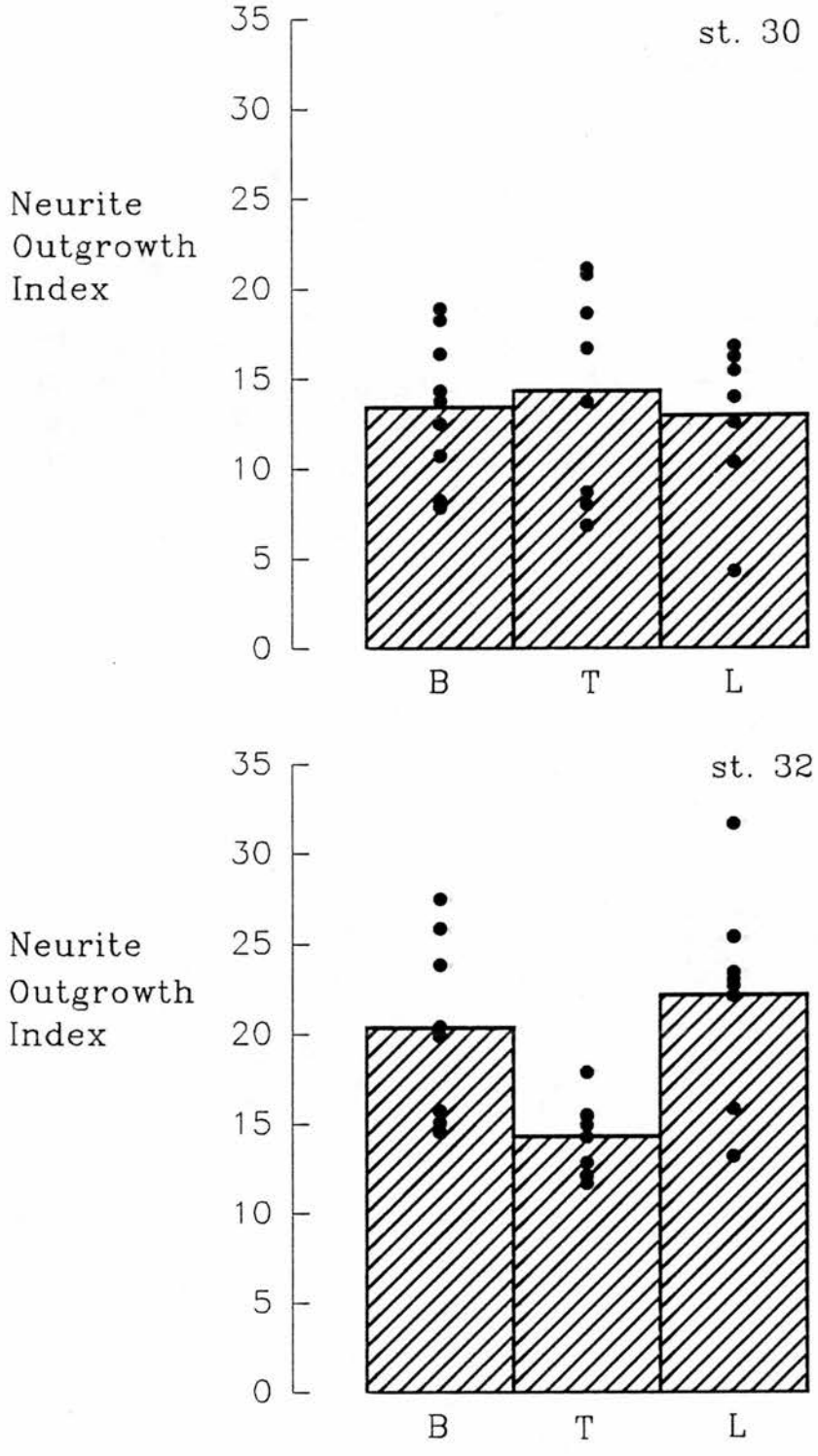


Fig.5.6

Neurite outgrowth after 24 h of culture from dorsal root ganglia isolated from stage 30 and 32 embryos. Bar height represents mean of explant scores for brachial (B), thoracic (T) and lumbosacral (L) ganglia, and data points are also shown.

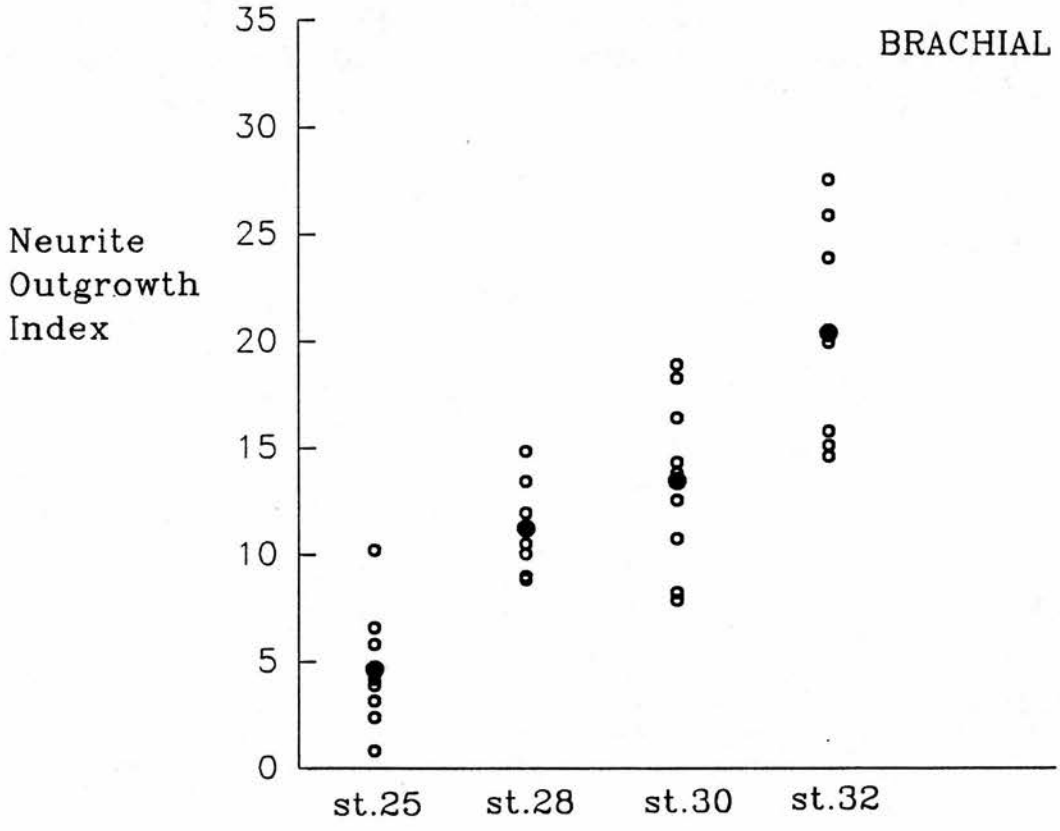


Fig.5.7

Effect of age on neurite outgrowth from brachial ganglia after 24 h of culture. Means (filled circles) and data points (open circles) are shown.

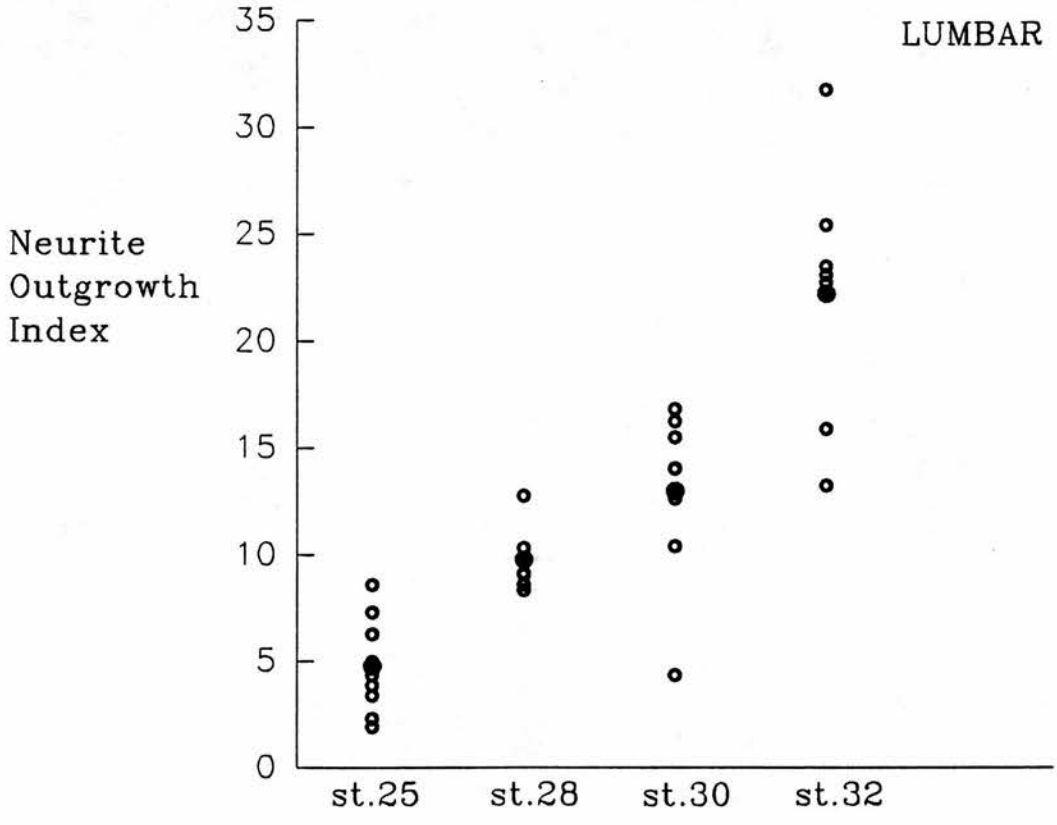


Fig.5.8

Effect of age on neurite outgrowth from lumbosacral ganglia after 24 h of culture. Means (filled circles) and data points (open circles) are shown.

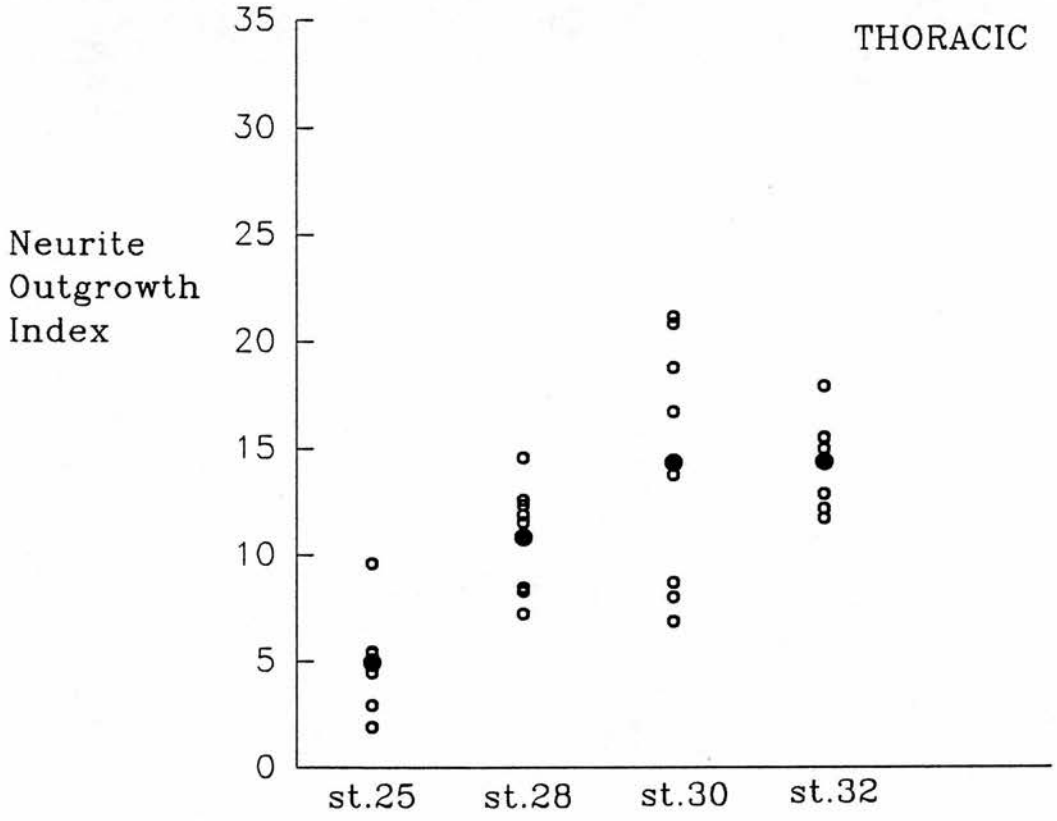


Fig.5.9

Effect of age on neurite outgrowth from thoracic ganglia after 24 h of culture. Means (filled circles) and data points (open circles) are shown.

DISCUSSION

The distance sensory axons must grow from dorsal root ganglia to reach their peripheral targets varies along the axis of the embryo. For example, sensory axons innervating the limbs grow considerably further than those innervating the trunk (Swanson & Lewis 1982). There is evidence that target distance may be related to growth rate: axons projecting over longer distances grow faster (Davies 1989a). The projection of axons from sensory neurons to peripheral targets could be controlled by intrinsic determination, environmental guidance or a balance of the two. Experiments were carried out to determine whether the differential axonal growth rates seen *in vivo* are the expression of an intrinsic neuronal property.

DRG isolated from brachial, trunk and lumbosacral segmental levels were cultured at an air-liquid interface. After 24 h no segmental differences were found in the amount of neurite outgrowth from ganglia taken from st.25, st.28, and st.30 embryos. This result supports that of Parson (1990) using dissociated neurons cultured on laminin or fibronectin. These data also support studies *in vivo* which demonstrate the ability of the limb bud to control axonal growth. If experiments are carried out in which the normal spatial relationship between DRG and the limb is perturbed, the pattern of innervation is often near normal (for example, Hamburger 1939; Swanson & Lewis 1982; Honig *et al* 1986). Experiments involving removal of specific tissues within the limb, such as ectoderm, muscle or motoneurons generally result in abnormal patterns of innervation (for example, Martin *et al* 1989; Lewis *et*

al 1981; Landmesser & Honig 1986; Swanson & Lewis 1986). In all cases the precise pattern of innervation is regulated by the limb.

There is also evidence that the rate at which DRG neurons project to their peripheral targets may also be determined by target properties. Swanson & Lewis (1982) studied wing bud innervation in silver stained whole mounts of chick embryos. At st.26 they found a clear difference in the lengths of axons invading limb and non-limb regions. Neurons which had entered the proximal environment of the limb were considerably longer. Such differences were even apparent between axons projecting from the same ganglion. However, this result does not preclude the possibilty that growth rates are intrinsically controlled as segmental gene expression would not necessarily be constrained by the limits of the ganglia.

Davies (1989a) showed that the growth rates of cranial sensory neurons are intrinsically determined. The ontogeny of cranial sensory ganglia and dorsal root ganglia may provide a partial explanation for the different mechanisms controlling axonal growth rates. Dorsal root ganglia are derived exclusively from neural crest cells (Le Douarin 1982). Lineage analysis *in situ* and *in vitro* has revealed the pluripotent and highly plastic nature of these cells which form many different structures in the adult (Bronner-Fraser & Fraser 1988; Baroffio *et al* 1988). It is not too surprising therefore to find that DRG neuronal growth rates are not intrinsically determined. The ontogeny of cranial sensory ganglia is rather more complex. They originate from both neural crest and ectodermal placodes, with individual ganglia receiving differing amounts from each source (D'Amico-Martel & Noden 1983; Davies & Lumsden 1990). The plasticity of placodal cells has not been investigated experimentally, although the

only tissues to which they contribute are sensory ganglia (D'Amico-Martel & Noden 1983; Narayanan & Narayanan 1980). This suggests they may follow a more strictly controlled growth program and it is not then surprising that the growth rates of cranial sensory ganglia are more strictly regulated.

Two types of guidance cue exist in the embryonic chick limb. General navigational cues enabling invading growth cones to trace out the gross anatomical nerve pattern and more specific cues directing the formation of specific connections (Swanson & Lewis 1982; Landmesser 1984,1991; Westerfield & Eisen 1988). In addition, the present study implies the presence of signals that stimulate axonal growth rate. The molecular nature of specific guidance cues is unknown. There is evidence however that common pathways are delineated by extracellular matrix molecules and these may also influence axonal growth rates.

Laminin and fibronectin are effective substrates for growth and differentiation of DRG neurons *in vitro* (Rogers *et al* 1983; Carbonetto *et al* 1983; Ernsberger & Rohrer 1988). A number of studies has shown that outgrowth on fibronectin is considerably less than on laminin despite increased adhesion on fibronectin (Rogers *et al* 1983; Carbonetto *et al* 1983; Gundersen 1987). Immunohistochemical studies *in vivo* have demonstrated that laminin is present at early developmental stages (st.13-st.25) along the trajectories of sensory axons emerging from DRG and entering the proximal regions of the limb buds (Rogers *et al* 1986). The presence of laminin in the chick limb at later developmental stages and along the pathways of developing nerves has not been shown. The distribution of fibronectin in the chick periphery was investigated by Yip &

Yip (1990). They found that fibronectin was distributed as a meshwork throughout limb and non-limb mesenchyme during axonal outgrowth but there was no preformed fibronectin pathway on which axons grew. This result suggests that fibronectin does not direct peripheral nerves towards their targets, but may however provide a permissive substrate. The distribution of fibronectin in both limb buds and trunk make it an unlikely candidate molecule for stimulating axonal growth rates in developing limb.

Permissive substrate molecules such as fibronectin and laminin, and inhibitory molecules such as chondroitin sulphate proteoglycan and PNA-binding glycoproteins define the system of general pathways or highways in the limb. It is over this system that more complex guidance mechanisms and neurotrophic interactions may operate (Landmesser 1991; Davies 1987). The factor responsible for increased axonal growth rates in the limb will operate against this backdrop. The survival of sensory neurons and outgrowth of neurites is regulated by neurotrophic factors produced in peripheral and central target fields (Davies 1988; Davies & Lumsden 1990). It is possible that increased axonal growth rates in the chick limb bud are mediated by a soluble target-derived factor. Sensory neurons isolated prior to target contact survive independently of NGF, BDNF and NT-3 *in vitro* (Davies & Lumsden 1984; Vogel & Davies 1991), although this does not preclude the possibility that neurotrophic factors affect axonal growth rates. NGF receptor mRNA has been detected in chick limb buds during early stages of axonal growth prior to target contact (Vogel & Davies 1991).

In addition to the finding that there is no segmental difference in the amount of neurite outgrowth from DRG isolated from st.25-st.30 ganglia

(E 4.5-E 6.5), the present study revealed that segmental differences do exist for older ganglia, st.32 (E 8). The reason for this is unclear. A neuron's regenerative capabilities may depend on its characteristics *in vivo*, such as axonal length or target innervation status. Sensory neurons are originally neurotrophic factor independent and become dependent on neurotrophic factors for survival only after target contact (Vogel & Davies 1990; Wright *et al* 1992). The time course of target innervation and therefore of neurotrophic factor dependence, may differ segmentally. Not much is known about target innervation in the trunk. Saxod (1978) states that the first cutaneous nerve branches have reached the skin by E 4, although Verna & Saxod (1979) state that this does not occur until E 5-6. This is earlier than in limb buds where cutaneous nerve fibres do not appear until E 6 (Visintini & Levi-Montalcini 1939; Swanson & Lewis 1982) and the final pattern of cutaneous innervation around E 7.5 (Martin *et al* 1989; Swanson & Lewis 1982; Roncali 1970). Innervation of muscle spindles in limb buds occurs even later. They are first detected by histological methods at E 9 (Tello 1922) and stretch reflexes are first seen at E 10 (Visintini & Levi-Montalcini 1939). No information could be found on development of muscle innervation in chick trunk. Therefore, by E 8 trunk DRG contain neurons which have innervated cutaneous, and possibly muscle, targets about 1.5 days prior to those in limb DRG. This difference may account for the segmental differences in growth seen *in vitro* as trunk DRG may contain more neurotrophic factor-dependent neurons. Although NGF is present in the culture medium additional factors may be necessary to maintain neuronal viability. At E 6 the final pattern of sensory innervation may not yet be established on the trunk. Thus there may be more neurotrophic factor-independent neurons at this stage and cosequently segmental differences are not seen.

CHAPTER 6

INTRODUCTION

During the early stages of the development of sensory innervation axons project from dorsal root ganglia to the proximal mesenchyme of the limb bud. Here they stop and form a plexus with developing motoneurons and at stage 25 begin to move into the periphery. What controls the timing and direction of this outgrowth of axons from the plexus towards peripheral targets? The possibility that a peripherally derived attractant or growth enhancing factor determines this response was tested by co-culturing dorsal root ganglia with target tissue explants in close proximity on a PLYS substrate. PLYS was chosen as it does not have an independent neurite growth stimulating activity like, for example laminin or collagen.Cultures were incubated in medium which was serum-free and NGF-free.

Segmental differences in the rate of growth of axons from dorsal root ganglia into the periphery are not intrinsically determined (see previous chapter). The ability of axons invading developing limb buds to elongate more rapidly than axons projecting towards non-limb targets must therefore result from extrinsic signals within the limb bud. Some experiments were carried out with DRG and target tissue explants from different segmental levels in co-culture to test the efficacy of limb bud versus non-lmib bud target fields in eliciting neurite outgrowth.

RESULTS

The earliest neurite outgrowth from brachial dorsal root ganglia

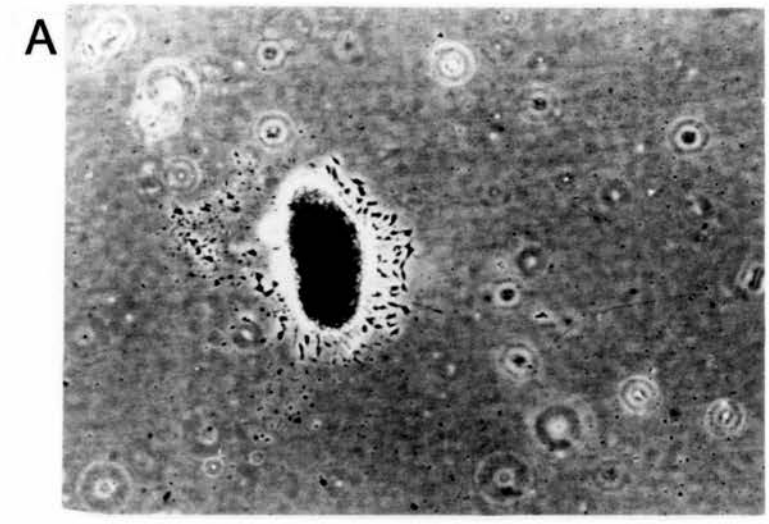
Brachial dorsal root ganglia isolated from stage 25 embryos were cultured with explants of stage 25 limb bud ectoderm, mesenchyme, heart and spinal cord (Figs.6.1 and 6.2). In wells containing DRG alone there was little neuronal growth (mean neurite outgrowth index=0.129; n=14; Fig.6.3). When ganglia were cultured with explants of ectoderm neuronal elongation was greatly stimulated (mean neurite outgrowth index=0.511; n=15; Wilcoxon unpaired test, $P<0.01$; Fig.6.3). In contrast, outgrowth was unaffected when ganglia were cultured with mesenchyme explants (mean neurite outgrowth index=0.132; n=8; $p>0.05$; Fig.6.3), heart explants (mean neurite outgrowth index=0.17; n=6; $p>0.05$; Fig.6.3), or spinal cord explants (mean neurite outgrowth index=0.0765; n=6; $p>0.05$; Fig.6.3).

The amount of neurite outgrowth from ganglia cultured with ectodermal explants was not influenced by the distance between explants (range, 0.1 mm to 1.8 mm; correlation coefficient, -0.002; Fig.6.4). Nor was the outgrowth of neurites directed specifically towards the ectodermal explant or away from it (Fig.6.5).

Brachial dorsal root ganglia isolated from stage 28 embryos responded to explants of limb bud ectoderm, mesenchyme and heart in a similar way to stage 25 ganglia (Figs.6.6 and 6.7). There was little or no outgrowth from these ganglia cultured alone (mean neurite outgrowth index=0.403; n=6; Fig.6.8) or in the presence of mesenchyme (mean neurite outgrowth index=0.27; n=6; Fig.6.8) or heart explants (mean neurite outgrowth

Fig.6.1

Stage 25 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG alone. B, DRG co-cultured with heart explant. Calibration bar, 500 μm.


A

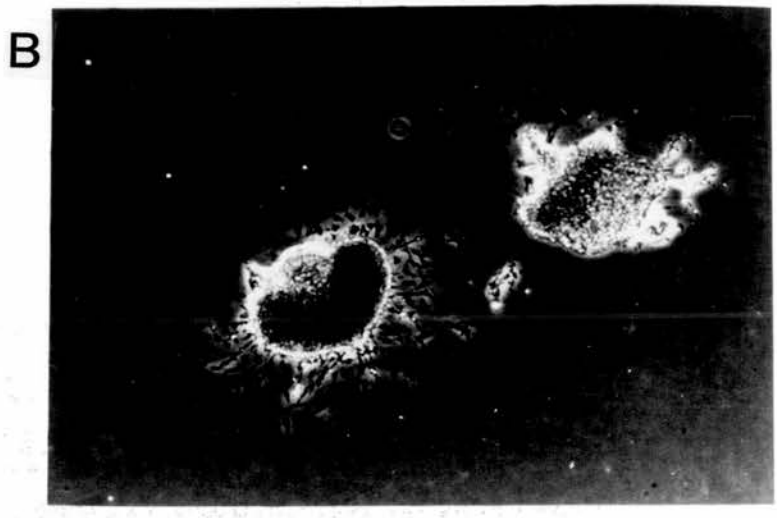
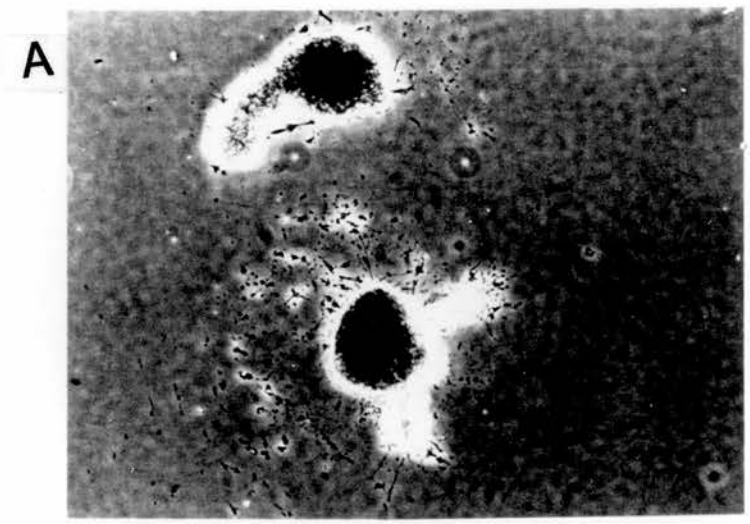
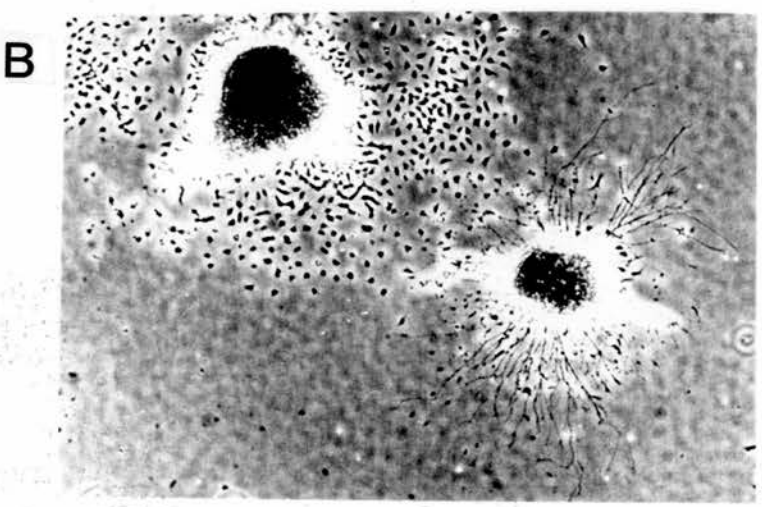

B

Fig.6.2

Stage 25 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG co-cultured with brachial mesodermal explant. B, DRG co-cultured with brachial ectodermal explant. Calibration bar, 500 μm.


A


B

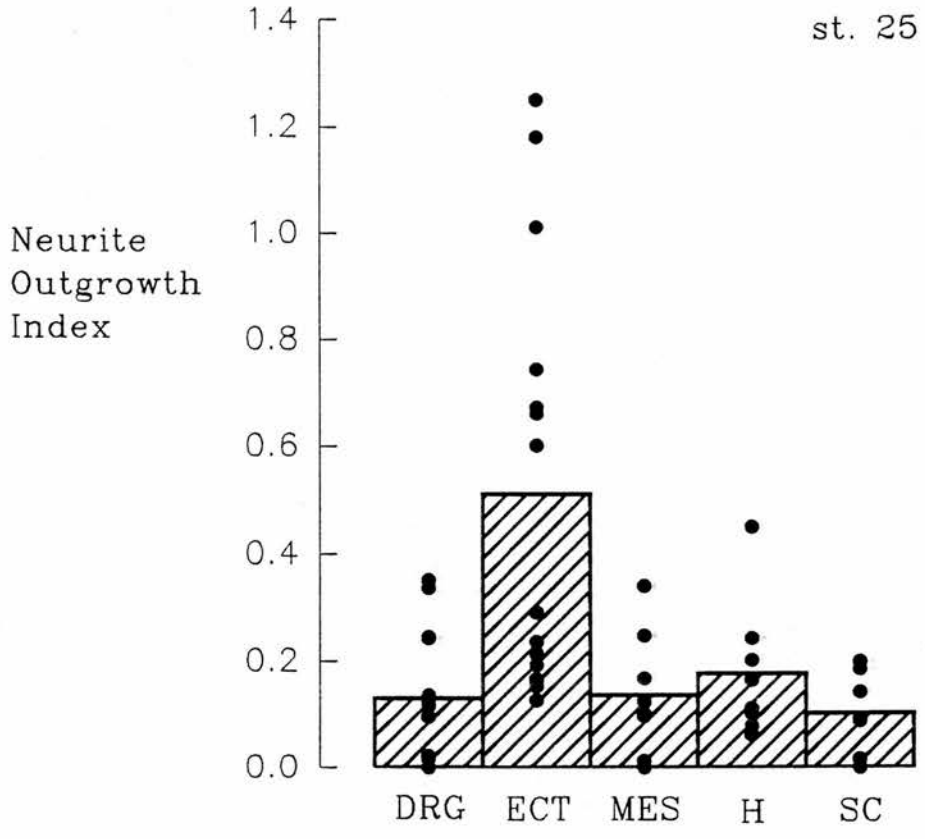


Fig.6.3

Bar chart showing the neurite outgrowth index for stage 25 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. Bar height represents the mean of the explant scores, and data points are also shown. DRG, dorsal root ganglion alone; ECT, dorsal root ganglion co-cultured with ectoderm; MES, dorsal root ganglion co-cultured with mesoderm; H, dorsal root ganglion co-cultured with heart; SC, dorsal root ganglion co-cultured with spinal cord.

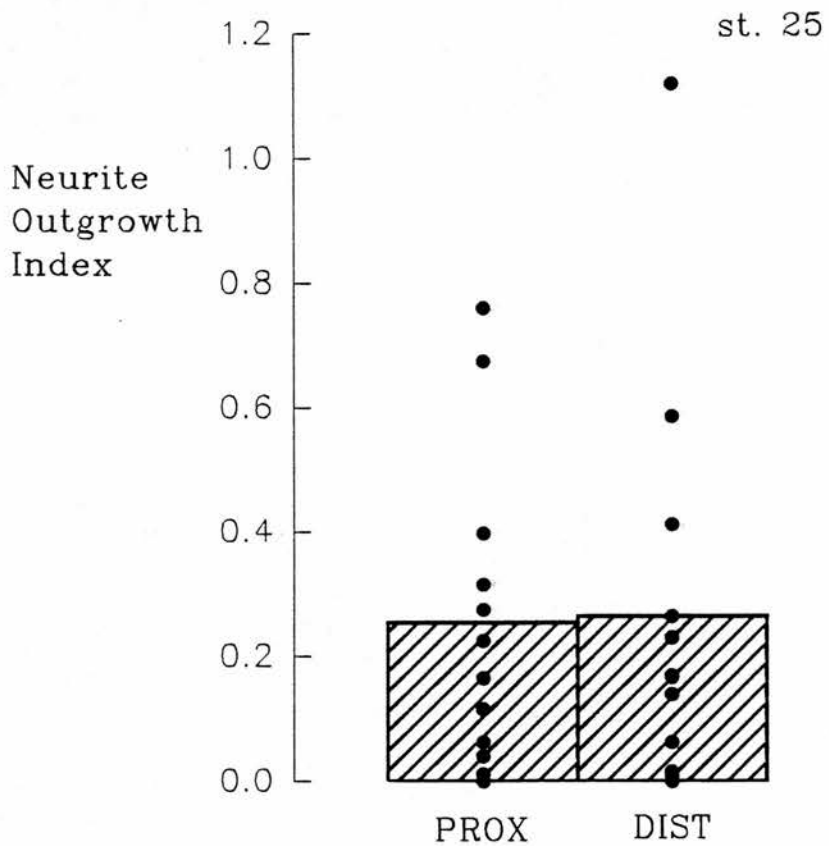


Fig.6.4

Bar chart showing the neurite outgrowth index for stage 25 brachial DRG co-cultured with ectodermal explants for 24 h. Bar height represents the mean of the explant scores and data points are shown. PROX, amount of outgrowth towards the ectodermal explant; DIST, the amount of outgrowth away from the ectodermal explant.

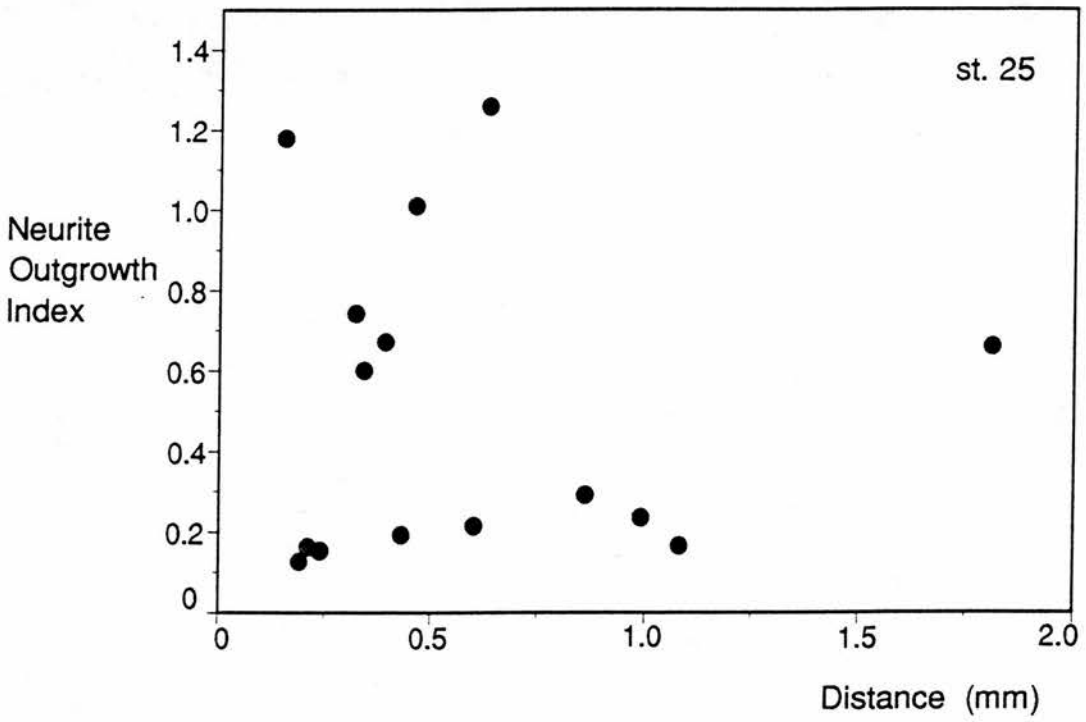
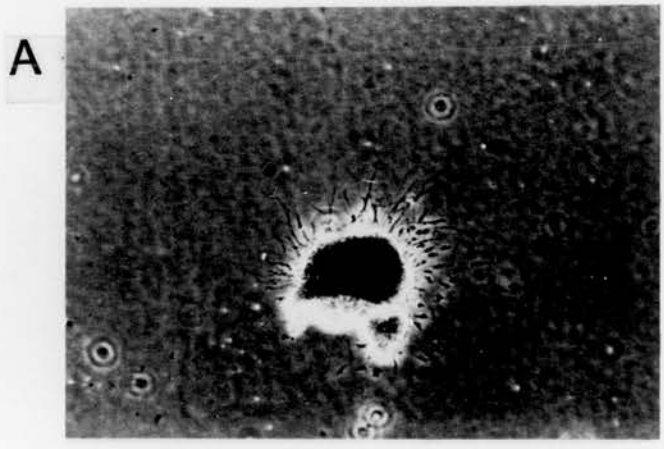


Fig.6.5

Effect of DRG to target distance on neurite outgrowth index for stage 25 brachial DRG co-cultured with ectodermal explants.Neurite outgrowth index is plotted against distance in mm.

Fig.6.6

Stage 28 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG alone. B, DRG co-cultured with heart explant. Calibration bar, 500 μm.


A

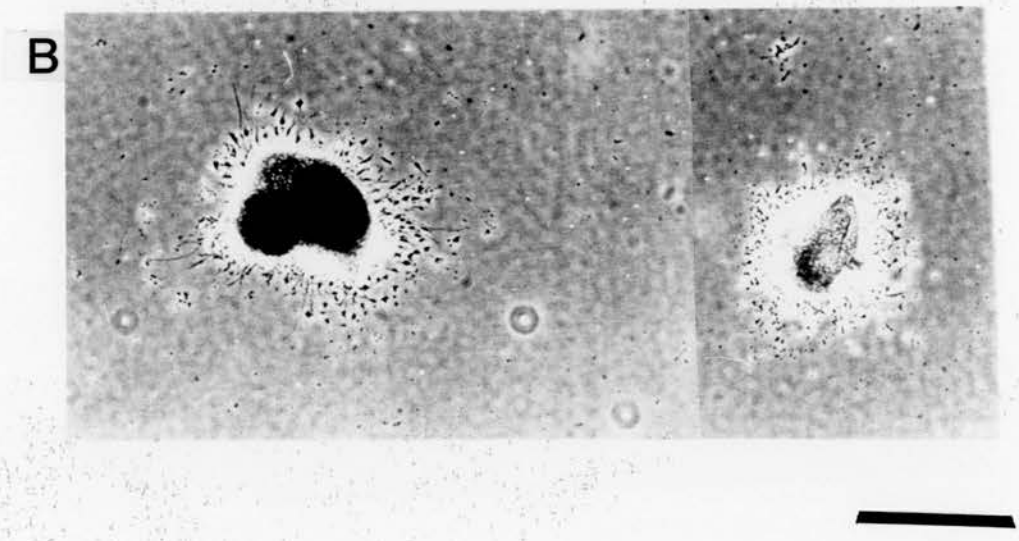
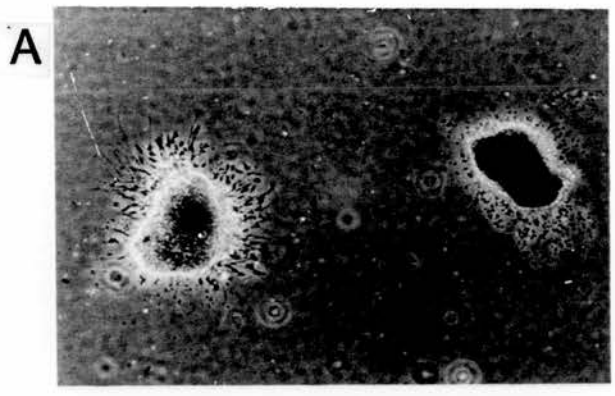
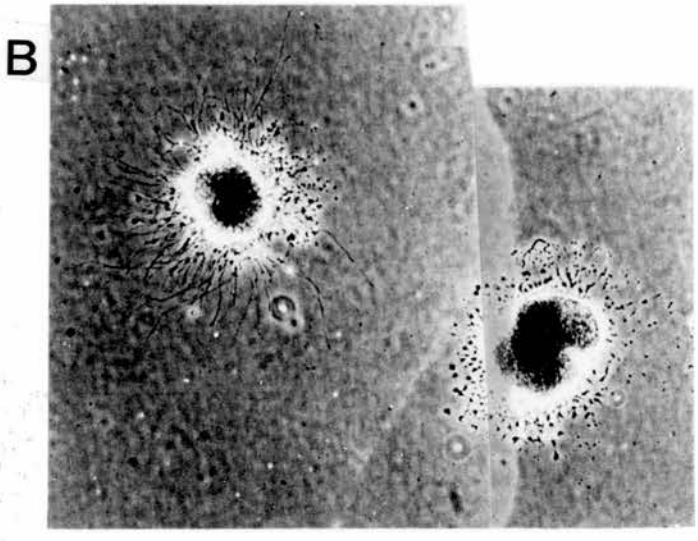

B

Fig.6.7

Stage 28 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG co-cultured with mesodermal explant. B, DRG co-cultured with ectodermal explant. Calibration bar, 500 μm


A


B

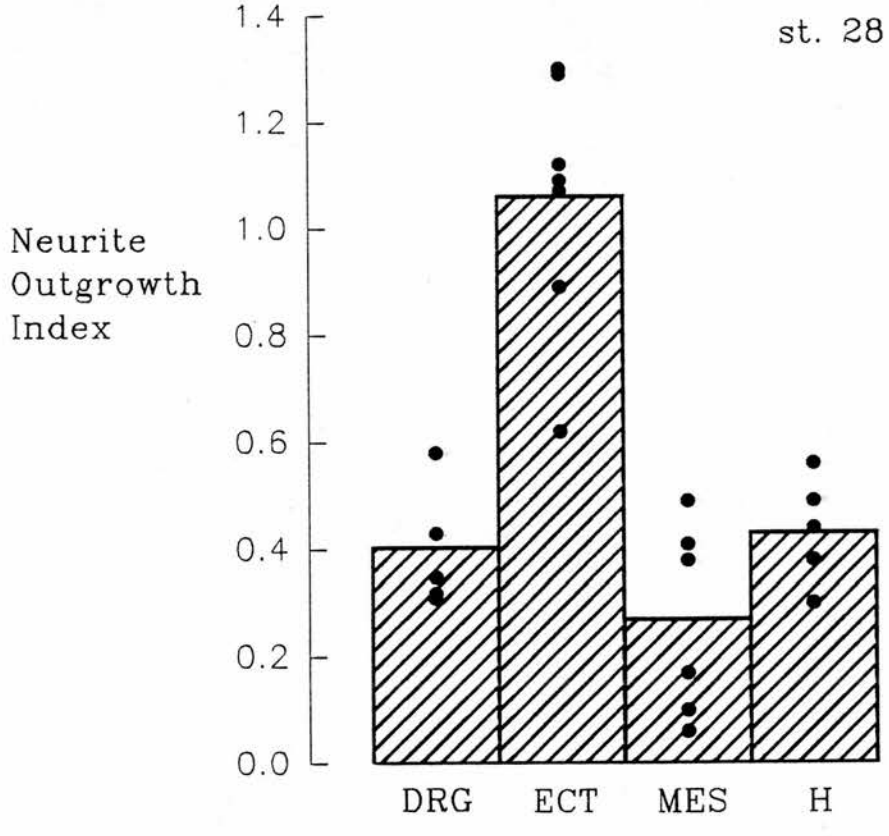


Fig.6.8

Bar chart showing the neurite outgrowth index for stage 28 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. Bar height represents the mean of the explant scores, and data points are also shown. DRG, dorsal root ganglion alone; ECT, dorsal root ganglion co-cultured with ectoderm; MES, dorsal root ganglion co-cultured with mesoderm; H, dorsal root ganglion co-cultured with heart.

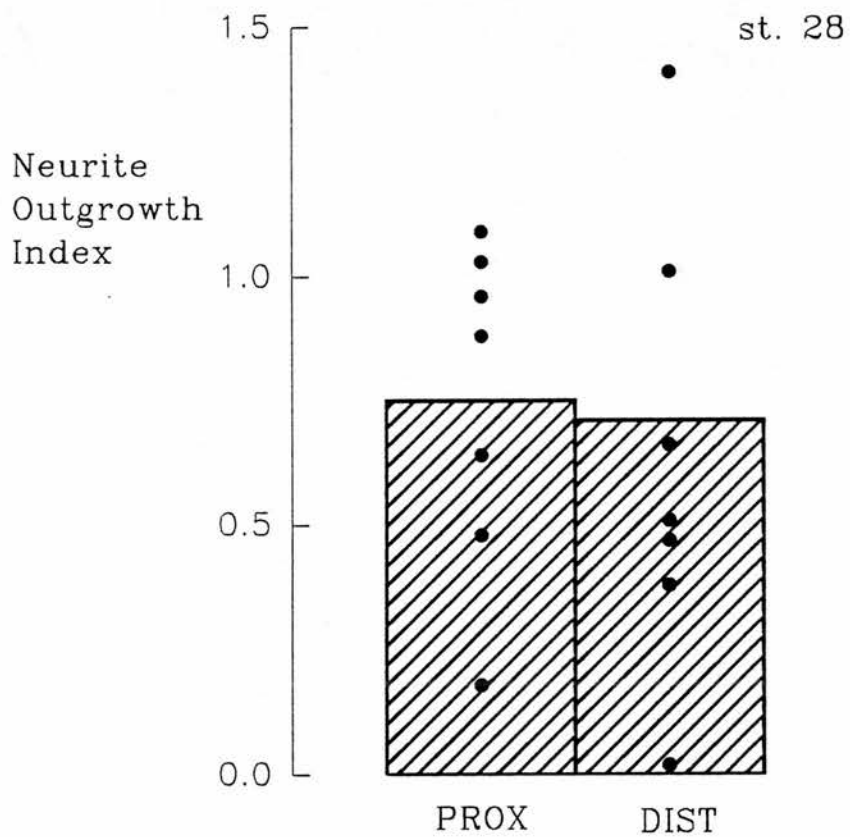


Fig.6.9

Bar chart showing the neurite outgrowth index for stage 28 brachial DRG co-cultured with ectodermal explants for 24 h. Bar height represents the mean of the explant scores and data points are shown. PROX, amount of outgrowth towards the ectodermal explant; DIST, the amount of outgrowth away from the ectodermal explant.

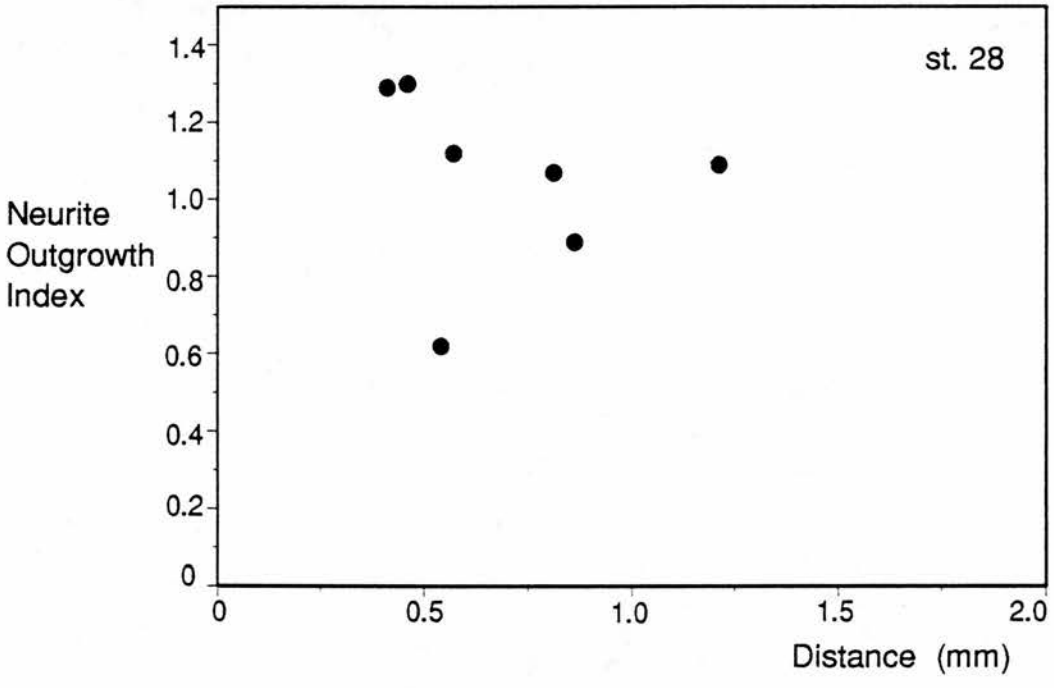


Fig.6.10

Effect of DRG to target distance on neurite outgrowth index for stage 28 brachial DRG co-cultured with ectodermal explants. Neurite outgrowth index is plotted against distance in mm.

Index=0.43; n=5; Fig.6.8). However, outgrowth was greatly stimulated when ganglia were cultured with ectodermal explants (mean neurite outgrowth index=1.06; n=7; Wilcoxon unpaired test, $P<0.05$; Fig.6.8). The amount of outgrowth was not affected by the distance between explants (range, 0.1-1.1 mm; correlation coefficient, -0.13; Fig.6.9) and was not directional (Fig.6.10).

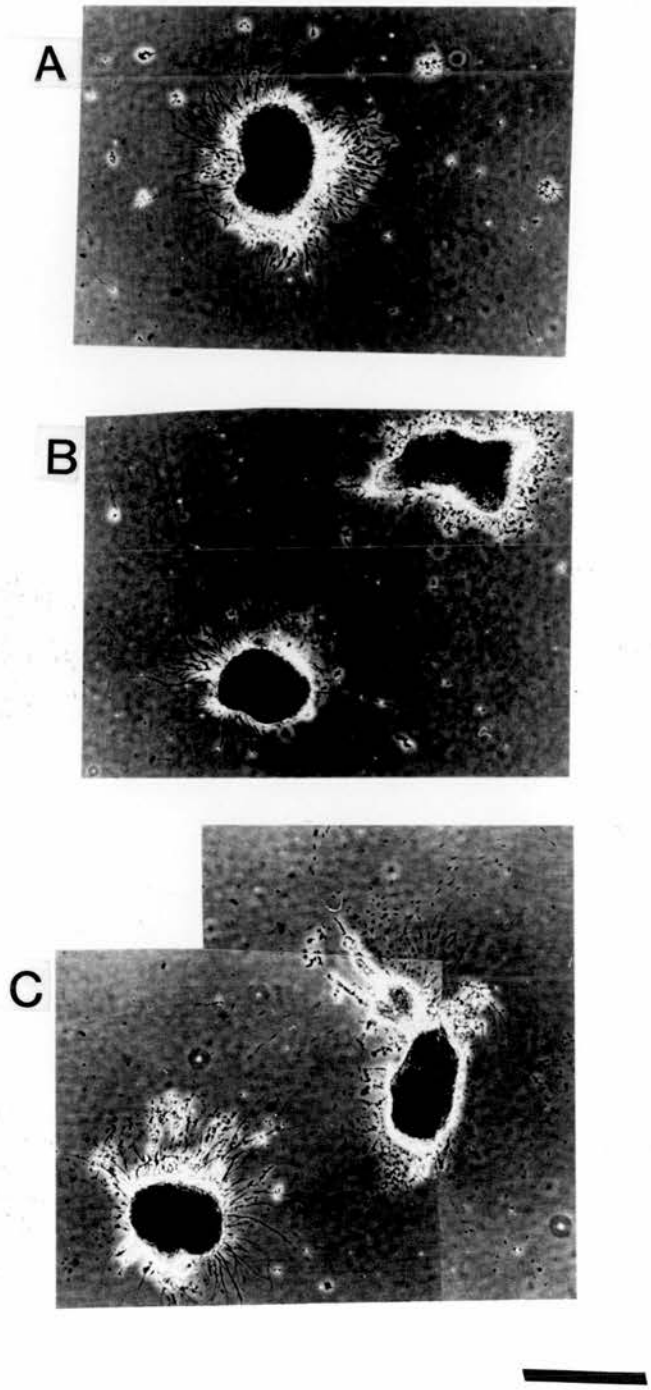
Neurite outgrowth at later developmental stages

Brachial dorsal root ganglia from stage 30 embryos were cultured alone or with explants of stage 30 limb bud ectoderm (Figs.6.11A and B). Substantial neurite outgrowth occurred from DRG cultured alone (neurite outgrowth index=0.466; n=7; Fig.6.12), and was not statistically different in the presence of ectoderm (neurite outgrowth index=0.801; n=8; Wilcoxon unpaired test $P>0.05$; Fig.6.12).

This result could be the effect of age related changes in the ectoderm or in the neurons. The ectoderm may no longer produce the growth stimulating factor, or the neurons may no longer require ectodermally derived neurotrophic support. To determine whether this effect is a property of the neurons, ganglia from stage 30 embryos were cultured with stage 25 limb bud ectoderm which stimulates growth of neuronal processes from stage 25 ganglia. No stimulation of outgrowth from stage 30 DRG was observed (neurite outgrowth index = 0.543; n=8; Wilcoxon unpaired test, $P>0.05$; Figs.6.11C and 6.12).

Fig.6.11

Stage 30 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG alone. B, DRG co-cultured with stage 30 ectodermal explant. C, DRG co-cultured with stage 25 ectodermal explant. Calibration bar, 500 μm.


A
B
C

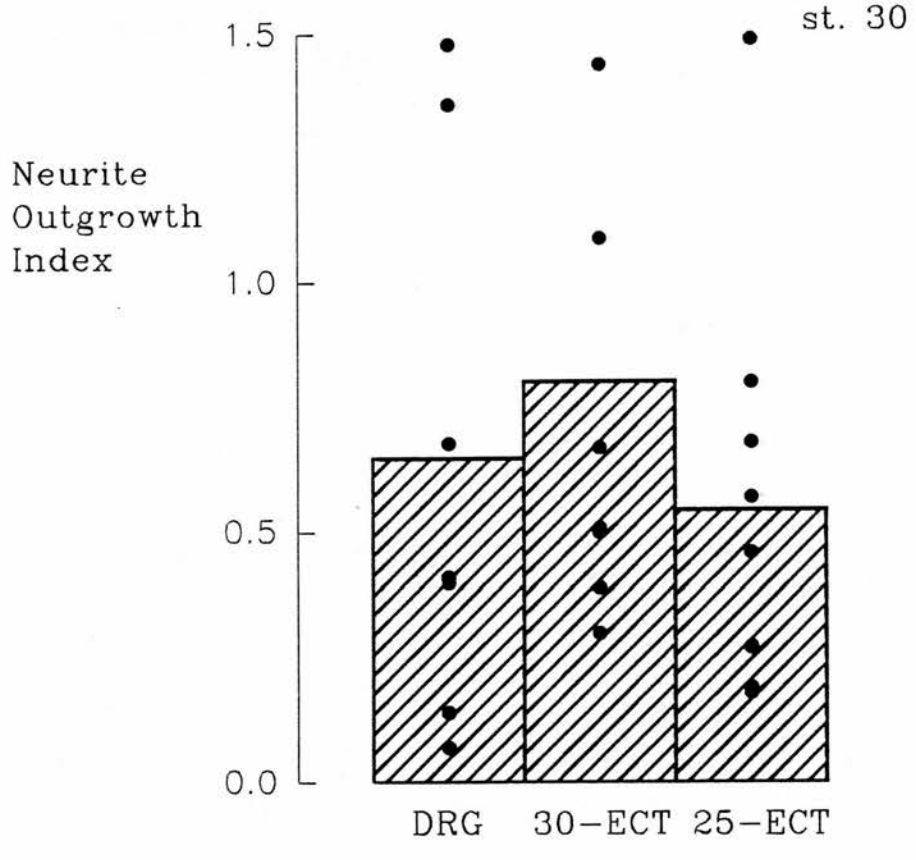


Fig.6.12

Bar chart showing the neurite outgrowth index for stage 30 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. Bar height represents the mean of the explant scores, and data points are also shown. DRG, dorsal root ganglion alone; 30-ECT, dorsal root ganglion co-cultured with stage 30 ectoderm; 25-ECT, dorsal root ganglion co-cultured with stage 25 ectoderm.

The earliest neurite outgrowth from thoracic dorsal root ganglia

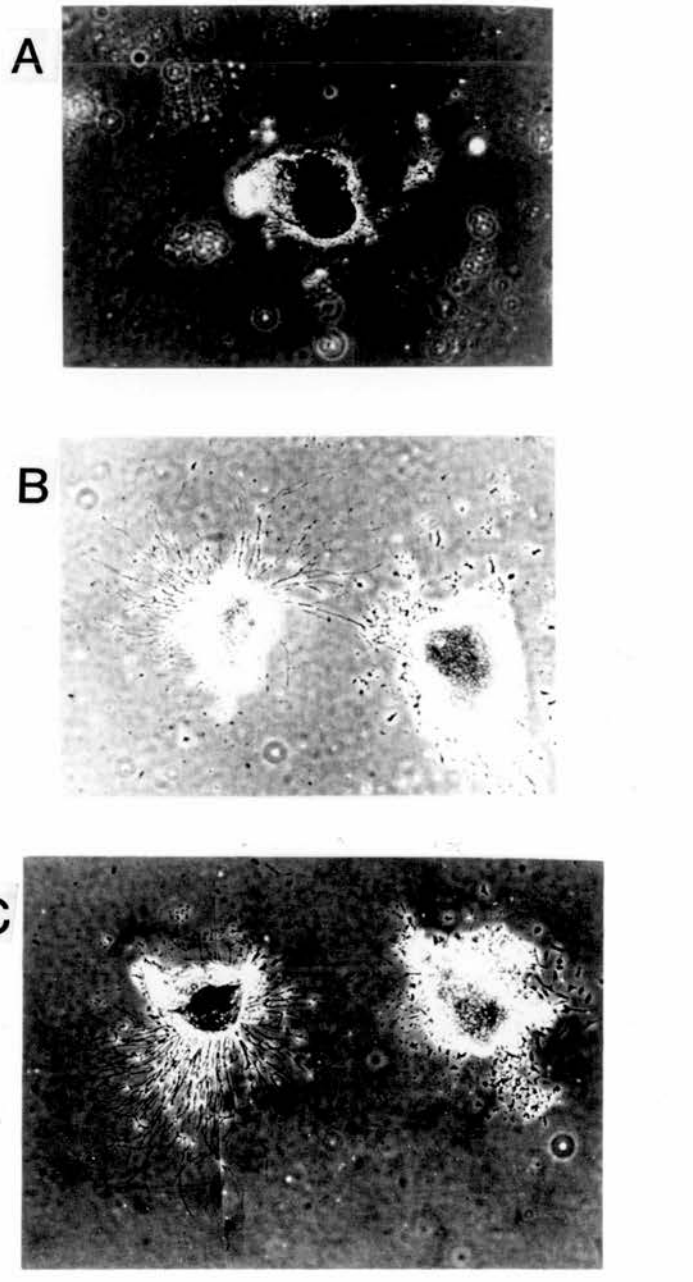
Thoracic dorsal root ganglia were cultured with explants of thoracic ectoderm and limb bud ectoderm (Fig.6.13). There was little or no neurite outgrowth from ganglia cultured alone (neurite outgrowth index=0.116; n=11; Fig.6.14), however, growth of neurons was stimulated by thoracic ectoderm (neurite outgrowth index=0.384; n=10; Wilcoxon unpaired test, $P<0.05$; Fig.6.14). In addition, thoracic DRG were cultured with explants of brachial ectoderm to determine whether segmental differences exist in the neurite growth stimulating capacity of early ectodermal tissue. Neurite outgrowth from thoracic DRG was stimulated by limb bud ectoderm (neurite outgrowth index=0.886; n=11; Fig.6.14) and the profuse outgrowth elicited was statistically significantly greater than outgrowth in the presence of thoracic ectoderm (Wilcoxon unpaired test, $P<0.05$).

The effect of medium conditioned by stage 25 ectoderm

To determine whether the neurite growth stimulating factor released by ectoderm is a soluble, diffusible factor, monolayers of dissociated ectoderm cells were cultured for three days and used to prepare conditioned medium. Stage 25 dorsal root ganglia were then cultured in control medium and in conditioned medium (Fig.6.15). Ectoderm conditioned medium stimulated neurite outgrowth dramatically (outgrowth index=1.22; n=6; Wilcoxon unpaired test, $P<0.01$; Fig.4.16) as compared with control ganglia (outgrowth index=0.148; n=7; Fig.4.16).

Fig.6.13

Stage 25 thoracic DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, DRG alone. B, DRG co-cultured with thoracic ectodermal explant. C, DRG co-cultured with brachial ectodermal explant. Calibration bar, 500 μm.


A
B
C

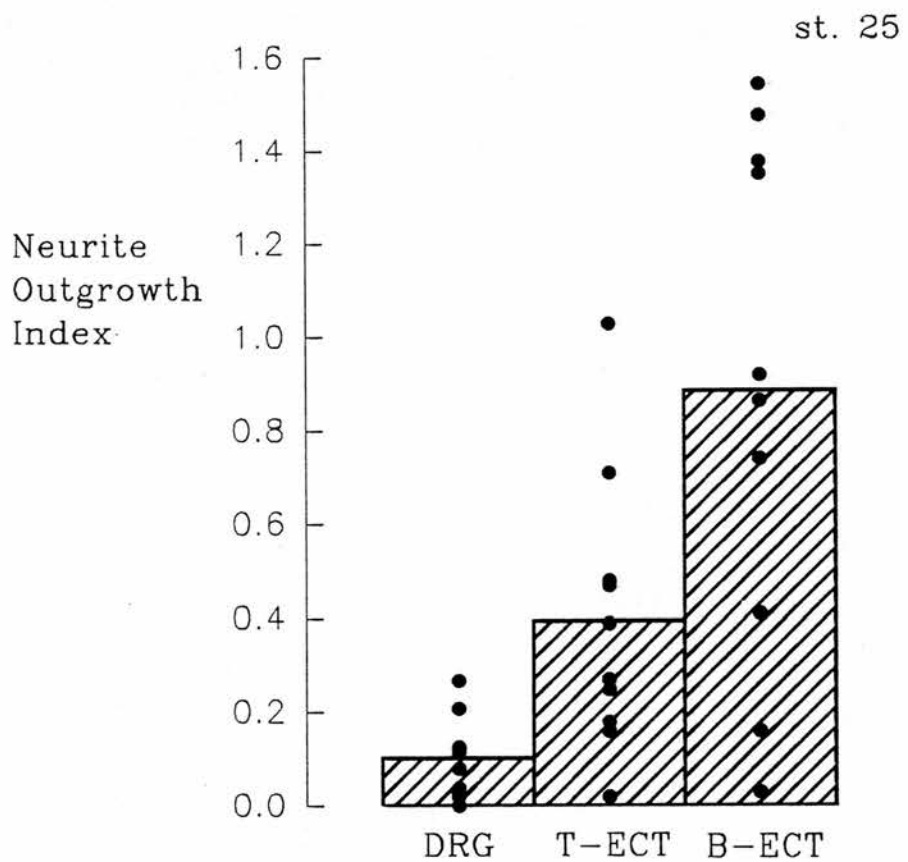
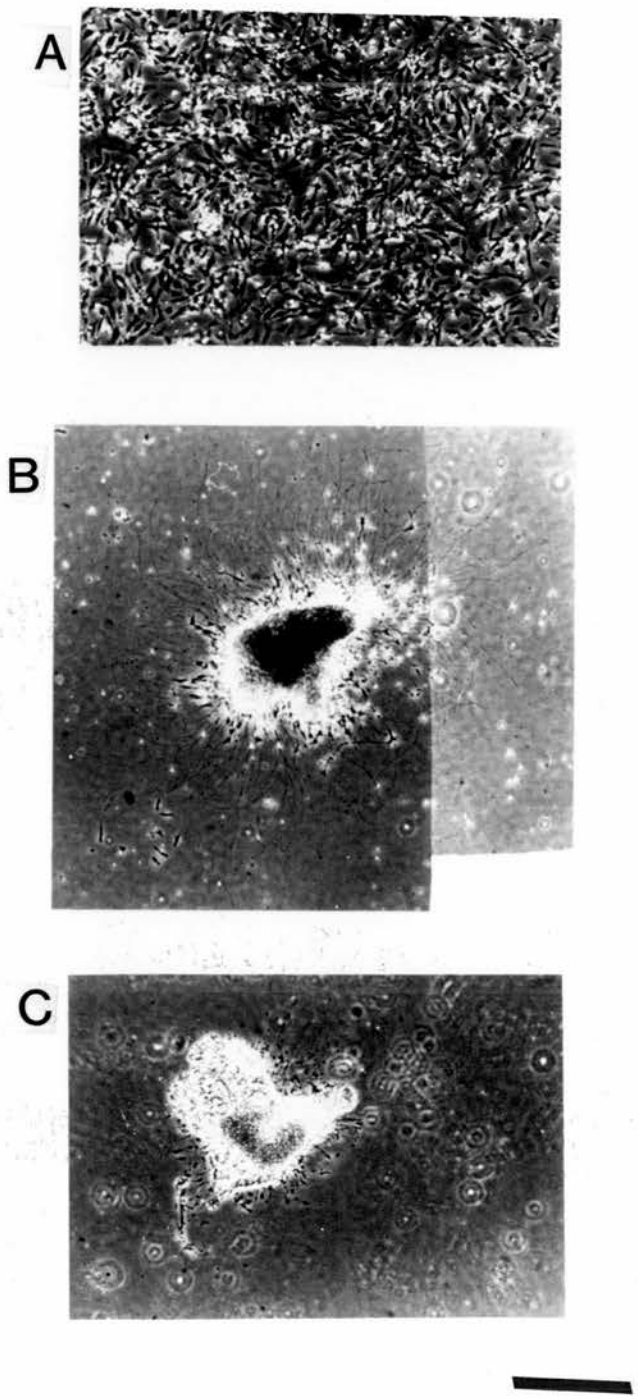


Fig.6.14

Bar chart showing the neurite outgrowth index for stage 25 thoracic DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. Bar height represents the mean of the explant scores, and data points are also shown. DRG, thoracic dorsal root ganglion alone; T-ECT, dorsal root ganglion co-cultured with thoracic ectoderm; B-ECT, dorsal root ganglion co-cultured with brachial ectoderm.

Fig.6.15

Effect of conditioned medium on neurite outgrowth from stage 25 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. A, monolayer of dissociated ectodermal cells after 3 days in culture. B, DRG after 24 h of culture in ectoderm-conditioned medium. C, DRG after 24 h of culture in control culture medium. Calibration bar, 500 μm.


A
B
C

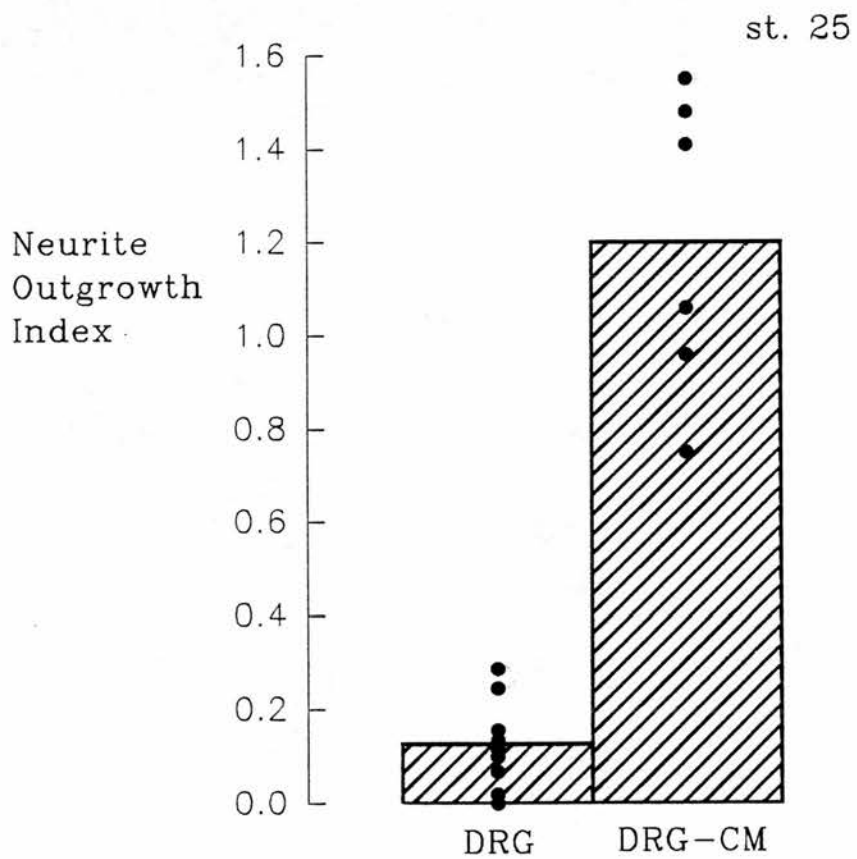


Fig.6.16

Bar chart showing the neurite outgrowth index for stage 25 brachial DRG explants after 24 h of culture on a PLYS coated substrate in serum-free, NGF-free medium. Bar height represents the mean of the explant scores, and data points are also shown. DRG, dorsal root ganglia cultured in control medium; CM, dorsal root ganglia cultured in ectoderm conditioned medium.

DISCUSSION

Target tissues or their products can influence neural development *in vitro*. This has been demonstrated for various parameters, ranging from the expression of neuron specific enzymes (Black 1978; Godfrey *et al* 1980) to stimulation of nerve fibre elongation (Ebendal & Jacobson 1977; Pollack *et al* 1981; Lumsden & Davies 1983, 1986; Tessier-Lavigne *et al* 1988). There is evidence that target tissues or their products control sensory innervation in the chick limb bud. Many studies *in vivo* have shown that the developing pattern of nerves in the limb bud is largely determined by limb tissues and is not an intrinsic property of the nerves themselves (Hamburger 1939; Swanson & Lewis 1982; Landmesser 1984; Scott 1987). Few studies have been made of the influence of limb tissues on sensory neurite outgrowth *in vitro* (Pollack *et al* 1980; Ebendal & Jacobson 1977), and none of these conducted using tissues from early developmental stages when sensory axons first begin to project into the limb *in vivo*.

Two aspects of the control of limb innervation have been investigated in the present study. Firstly, what controls the timing of initial axonal growth into the limb; and secondly what factors may be involved in promoting axonal growth rates in the limb. The results suggest that a soluble molecule derived from peripheral target tissue, particularly ectoderm, may be important.

Control of axonal outgrowth into the limb

During the early stages of sensory innervation, axons project from DRG to the proximal environment of the limb where they appear to " wait " for about 24 h before entering the limb proper (Bennet *et al* 1980; Hollyday 1983; Tosney & Landmesser 1985). During this period a plexus forms, facilitating axonal sorting, then at E 4.5 pioneer sensory and motor neurons begin to invade. What controls the timing of this initial projection? Landmesser (1987) proposed that axons are prevented from entering the limb due to the repulsive nature of early limb bud mesenchyme. Subsequently growth inhibiting molecules were identified in proximal limb mesenchyme by Oakley & Tosney (1991). Axonal penetration is correlated with loss of expression of inhibitory molecules. Perhaps the timing of neuronal projection into the limb is simply regulated by the growth inhibiting properties of early proximal mesenchyme. However, other studies have shown that the earliest outgrowth of some sensory axons towards their peripheral targets is stimulated by a soluble attractant (Lumsden & Davies 1983, 1986). Thus it is possible that early axonal growth into the limb bud is mediated by growth promoting as well as growth inhibiting signals.

This study presents a quantitative analysis of the influence exerted by peripheral target tissues on DRG neurite outgrowth in culture. Sensory axons invade the periphery prior to the differentiation of their targets: musculature and skin. Muscle differentiation occurs late in the time course of limb innervation, around E 9 (Lewis *et al* 1981). Sensory receptor differentiation has been detected as early as E6 by electrophysiological methods (Visintini & Levi-Montalcini 1939). Experiments were carried out

at E 4.5, E 5.5 and E 6.5. Consequently target tissue explants are described as mesenchyme and ectoderm rather than muscle and skin.

The present culture system utilized a serum- and extract-free medium, and a PLYS coated substrate. The reported observations may have gone undetected in serum-supplemented cultures with ganglia adhered to growth promoting substrate molecules such as laminin or fibronectin.

The results show that neurite growth from E 4.5 and E 5.5 ganglia was enhanced in the presence of limb bud ectoderm explants. At E 6.5 there was pronounced neurite outgrowth from ganglia cultured alone and this was not significantly increased by co-culture with ectoderm. Thus the stages of greatest response of DRG neurites to target presence *in vitro* were those nearest to the time when limbs are first invaded by pioneer axons *in vivo* (Al-Ghaith & Lewis 1982; Swanson & Lewis 1982). The developmental targets for sensory axons are immature tissues whose differentiation may create an environment for the optimal extension of nerve fibres. At E 6.5 some target innervation has occurred (this is the earliest stage at which spinal motor reflexes can be elicited by cutaneous stimulation; Visintini & Levi-Montalcini 1939), and ectoderm from this stage will not enhance neurite growth in the way that younger ectoderm enhances outgrowth from younger ganglia. Either the growth promoting properties of the ectoderm has been lost or the neurons are no longer receptive. The finding that ectoderm from E 4.5 embryos will not enhance outgrowth from E 6.5 DRG suggests that the neurons themselves have lost those properties which permited the enhanced neurite growth response. Pollack & Muhlach (1981) noted a similar age-dependent loss of

responsiveness of spinal cord tissues to limb bud mesenchyme in amphibia.

At none of the developmental stages studied did limb mesenchyme tissue affect DRG neurite outgrowth. However, Pollack *et al* (1980) showed that outgrowth from amphibian DRG was stimulated by limb bud mesenchyme in co-culture. In those experiments explants were taken after innervation had occurred and trophic interactions were established. Newly differentiated muscle produces BDNF which can support the growth and survival of both sensory and motor neurons *in vivo* and *in vitro* (Oppenheim *et al* 1992, Davies *et al* 1986, Barde *et al* 1987). In the present study, DRG were isolated at E 4.5, before they had started to invade the limb, and at E 5.5, during the initial stage of growth into the limb. Experiments have shown that no target innervation exists at these stages (Visintini & Levi-Montalcini 1939; Lewis *et al* 1981; Swanson & Lewis 1982).

The ventrolateral cells of the DRG differentiate much earlier than the dorsomedial cells (Hamburger & Levi-Montalcini 1949; Jacobson 1978; Hamburger *et al* 1981). Consequently the first axons to invade the limb bud at E 4.5 project from ventrolateral neurons. Dorsomedial cells do not form a significant population until E 6 (Hamburger *et al* 1981) and the bulk of DM axonal outgrowth will therefore occur after this stage. Visintini & Levi-Montalcini (1939) elicited spinal motor reflexes by cutaneous stimulation at E 6 suggesting that the first neurons to form cutaneous connections are ventrolateral neurons. Visintini & Levi-Montalcini (1939) and subsequently Hamburger & Levi-Montalcini (1949) proposed that cells within the DRG were functionaly segregated: ventrolateral neurons

forming cutaneous afferents; dorsomedial neurons forming muscle afferents. However, Scott (1982) showed by injecting the retrograde tracer HRP into chick limb buds that cutaneous and muscle afferents were not positionally segregated in the ganglia. Although the VL cell poulation does not consist entirely of cutaneous afferents, a significant proportion will be. Thus many of the earliest axons to invade the limb project to developing ectoderm. It is not therefore surprising that ectodermal tissues enhance the earliest neurite outgrowth from avian sensory ganglia.

A full understanding of the response of DRG to ectoderm explants *in vitro* would require the isolation and identification of the growth stimulating factor. This was beyond the scope of the present thesis. However, medium conditioned by cultures of dissociated ectodermal cells was found to enhance neurite outgrowth. The growth stimulating factor is therefore a soluble molecule secreted into the culture medium. This is expected as the enhanced neurite growth seen in co-culture occurs at a distance. The identity of the factor is unknown but the most obvious candidate molecules are the neurotrophic factors NGF, BDNF and NT-3 which have been shown to promote survival and stimulate neurite outgrowth from chick embryo DRG *in vitro* (Levi-Montalcini & Angeletti 1963; Barde *et al* 1982; Hohn *et al* 1990). However, many of these studies have focused on neurons that have already innervated their targets. Studies using sensory neurons isolated prior to target contact have shown that they do not require NGF, BDNF or NT-3 for survival at early stages (Davies & Lumsden 1984; Vogel & Davies 1991). Other types of neurons, for example early sympathetic neurons and retinal ganglion cells, are also independent of neurotrophic factors prior to target contact (Coughlin & Collins 1985; Rodriguez-Tebar *et al* 1989). Although sensory neurons do

not require neurotrophic factors for survival at early developmental stages they express mRNA for the low affinity neurotrophic factor receptor $gp75^{NGFR}$ from the earliest stages of axonal outgrowth (Heuer *et al* 1990; Wyatt *et al* 1991). Thus early sensory neurons can recognize NGF, BDNF and NT-3 before they become dependent on them for survival. It is possible that neurotrophic molecules direct the growth of early sensory neurons towards their peripheral targets without also being required for cell survival. In addition Wright *et al* (1992) have shown that at E 4.5 chick DRG neurons *in vitro* respond to BDNF and NT-3. At this early stage neurons undergo a morphological maturation from small, spindle shaped, phase dark cells to large, spherical, phase bright cells which extend neurites (Panesse 1972). BDNF and NT-3 accelerate this morphological maturation (Wright *et al* 1992).

NGF treatment was shown to prevent naturally occurring cell death *in vivo* (Hamburger *et al* 1981) and maintain the normal development of DRG cells following target tissue removal (Hamburger & Yip 1984). However, Straznicky & Rush (1985) found that the daily administration of NGF (at a higher concentration to that used by Hamburger & Yip) did not prevent cell death induced by target removal in the chick. This suggests that chick DRG neurons require in addition to NGF, other maintainance factors produced by the periphery. As well as functioning to maintain cell survival, these may also enhance and direct the initial outgrowth of axons into the limb.

Although diffusible factors, for example neurotrophins, are obvious candidate molecules for the growth stimulating activity described, it is possible that a different type of molecule is eliciting the response.

Extracellular matrix glycoproteins such as laminin and fibronectin may be secreted by many tissue explants *in vitro* (for example see Collins & Garrett 1980) and enhance outgrowth from adjacent ganglia by providing a compliant substrate or stimulating growth directly.

Molecules produced by limb ectoderm may enhance and possibly direct neurite outgrowth into the limb at critical developmental stages. Directed outgrowth was not observed in culture however. Orientation of growing neurites requires the formation of a gradient of attractant with cells nearest the target explant receiving more of the growth enhancing molecule, resulting in more profuse outgrowth in that direction. The establishment of a gradient in a two dimensional culture chamber may not be possible due to stirring of molecules released into the medium. Gradients can be set up by culturing tissues in three dimensional collagen gel matrices (for example Lumsden & Davies 1983, 1986; Tessier-Lavigne *et al* 1988). However, collagen can directly stimulate sensory neurite outgrowth (Carbonetto *et al* 1983) and is consequently unsuitable for the present study.

To summarize: the timing of the earlist sensory neurite outgrowth into the developing limb bud correlates with a soluble neurite growth stimulating activity in limb ectoderm. At later stages of development, when many neurons have contacted their targets, the growth stimulating properties of ectoderm are absent or reduced. This result suggests that the early invasion of axons into the limb bud may be regulated by an ectodermally derived growth stimulating factor in addition to the non-permissive propeties of proximal mesenchyme.

Axonal outgrowth at different segmental levels

DRG isolated from thoracic segmental levels were also cultured with explants of peripheral target tissue. The results were similar to those previously described using brachial DRG. Outgrowth was stimulated by explants of ectoderm isolated from E 4.5 and E 5.5 embryos, the stage at which axonal growth into the periphery begins. Non-permissive mesenchyme may also be present along the flanks of the embryo as well as in the proximal limb buds, although Oakley & Tosney (1991) did not examine this. Thus the regulation of the timing of sensory neuron outgrowth may be similar at all segmental levels.

Comparison of the amount of neurite outgrowth elicited from brachial and thoracic DRG by ectoderm of corresponding segmental origin showed that more outgrowth occurred from brachial than from thoracic ganglia. Indeed thoracic DRG cultured with explants of limb bud ectoderm showed significantly greater neurite outgrowth than when cultured with thoracic ectoderm. This difference in the growth stimulating properties of brachial and thoracic ectoderm may be related to the differences in axonal growth rates of neurons located at brachial and thoracic segmental levels. The increased growth rates of axons invading limbs may reflect a higher concentration of ectodermally derived growth stimulating activity.

It should be noted that experiments of the kind described above tell us little about the nature of the growth enhancing factor. In particular, it is not possible to determine whether the factor produced is a neurite promoting factor as such, or simply a neuronal survival factor. However, Wright *et al* (1992) have shown that E 4.5 DRG neurons will survive *in vitro* in the

absence of neurotrophic factors so perhaps survival factors are not required by neurons at this early stage.

GENERAL CONCLUSION

Neurite outgrowth in culture is most commonly studied after first attaching neural explants or dissociated cells to an adhesive substrate. In this thesis a novel tissue culture preparation using ganglia floating at the surface of serum-free culture medium has been introduced and described in detail. Once neurite elongation along the surface of culture medium has been described there are a number of routes which can be followed, all of which have the aim of discovering the intrinsic mechanisms involved in the growth process.

The exact nature of the adhesive contacts between the growth cone and interface is unknown, although the results presented in this thesis (chapters 3 and 4) point to a strong adhesive interaction perhaps based on the biophysical properties of the cell surface rather than on specific molecular interactions. Reducing surface tension, and therefore altering the surface potential, decreases the amount of neurite outgrowth. Cytoskeletal activity, on which the protrusive and contractile properties of the growth cone depend, was studied using drugs selective for different cytoskeletal elements (chapter 4). The results obtained using colcemid and nocodazole demonstrate that microtubule assembly is an absolute requirement for neurite extension. Thus a protrusive or " push " mechanism contributes to the motile process. The cytochalasin B results are more difficult to interpret. They suggest that although actin based processes such as filopodial extension and growth cone traction are not essential for growth, they do have a role. The overall conclusion that both microtubule assembly and actin based traction contribute to neurite

elongation is in agreement with most other studies (for example see Bray 1991; Goldberg & Burmeister 1988; Letourneau *et al* 1987). However, the properties of an air-liquid interface are such that it may not be possible for cellular traction and tension development to occur. Clearly further work needs to be carried out on the surface properties of culture medium.

Ganglia adhered to the extracellular matrix glycoprotein laminin were also cultured with cytochalasin B and microtubule disorganizing drugs. The results suggest laminin is a poorly adhesive substrate and therefore concur with the opinion that neurite elongation on laminin is directly stimulated by a receptor-mediated event (see Gundersen 1987; Bixby 1989).

In addition to raising many questions about the role of growth cone adhesion and traction of cytoskeletal proteins in the mechanism of axon outgrowth, the floating ganglion preparation also offers a number of advantages over conventional culture methods for addressing developmental questions. Some of these were described in the present thesis. Identification of novel neurotrophic factors relies on bioassay of growth factor activity *in vitro*. Many glycoproteins commonly used to coat culture substrata have an independent co-trophic effect on neurite outgrowth. It would obviously be helpful to disentangle the simultaneous effects of soluble and substrate-bound neurotrophic activity in order to obtain a clearer understanding of how neurite growth is controlled. The floating ganglion preparation offers the possibility to do this.

Neurite outgrowth is greater from floating ganglia than from those adhered to polylysine, so the floating ganglion preparation provides a more

sensitive assay for neurite growth. To determine whether segmental differences seen in the rates of neurite outgrowth *in vivo* are intrinsically determined or environmentally regulated, ganglia were isolated from different segmental levels and the amount of outgrowth measured after 24 h. It is obviously desirable that the culture environment does not contain molecules, such as laminin or fibronectin, that could act as neuronal growth regulators. Floating ganglia of differing segmental origin showed no differences in the amount of neurite outgrowth produced after 24 h of culture, suggesting that segmental differences in growth rate are environmentally imposed (see chapter 5) and this supports the findings of many studies *in vivo* (for example Swanson & Lewis 1982, 1986; Tosney & Landmesser 1985).

The regulatory effect of target tissues on sensory neurite outgrowth was addressed further. In these experiments more conventional culture methods were used as it was necessary to maintain explants in close proximity throughout a 24 h culture period. The results obtained suggest that the earliest sensory axons to grow into the periphery may be responding to a soluble ectoderm-derived trophic molecule (see chapter 6). Lumsden & Davies (1983, 1986) found evidence that other types of sensory neurons respond to ectoderm-derived growth stimulating molecules at early develomental stages. The identity of this growth promoting activity is not known and further work is needed to isolate and characterize the factor. If an ectodermally derived factor regulates the differential axonal growth rates seen at different segmental levels this would require either i) different quantities of the factor to be present in limb and non-limb target fields, ii) segmental differences in receptor expression by neurons, iii) different forms of the factor present at different segmental

levels. In this there is much scope for further work. Deciphering the mechanisms by which growing neurites interact with environmental cues will allow an understanding of how the proper functioning of the nervous system emerges during development.

BIBLIOGRAPHY

Acheson, A., Edgar, D. and Timpl, R. (1986) Laminin increases both levels and activity of tyrosine hydroxylase in calf adrenal chromaffin cells. J. Cell Biol. 102: 151-159.

Acklin, S.E. and Nicholls, J.G. (1990) Intrinsic and extrinsic factors influencing properties and growth patterns of identified leech neurons in culture. J. Neurosci. 10: 1082-1090.

Adam, N. K. (1938) The physics and chemistry of surfaces. OUP, Oxford, UK.

Akers, R.M., Mosher, D.F. and Lilien, J.E. (1981) Promotion of retinal neurite outgrowth by substratum-bound fibronectin. Dev. Biol. 86: 179-188.

Al-Ghaith, L.K. and Lewis, J.H. (1982) Pioneer growth cones in virgin mesenchyme: an electron microscope study in the developing chick wing. J. Embryol. Exp. Morph. 68: 149-160.

Bamburg, J.R., Bray, D. and Chapman, K. (1986) Assembly of microtubules at the tip of growing axons. Nature 321: 788-790.

Banker, G.A. and Cowan, W.M. (1979) Further observations on hippocampal neurons in dispersed cell culture. J. Comp. Neurol. 187: 469-494.

Barde, Y.A., Edgar, D. and Thoenen, H. (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J. 1: 549-553.

Barde, Y.A., Davies, A.M., Johnson, J.E., Lindsay, R.M. and Thoenen, H. (1987) Brain derived neurotrophic factor. Prog. Brain Res. 71: 185-189.

Baroffio, A., Dupin, E. and Le Douarin, N.M. (1988) Clone-forming ability and differentiation potential of migratory crest cells. Proc. Natl. Acad. Sci. USA 85: 5325-5329.

Baron-van Evercooren, A., Kleinman, H.K., Ohno, S., Marangos, P., Schwartz, J.P. and Dubois-Daiqc, M.E. (1982) Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8: 179-193.

Bastiani, M.J., Doe, C.Q., Helfand, S.L. and Goodman, C.S. (1985) Neuronal specificity and growth cone guidance in grasshopper and drosophila embryos. Trends Neurosci. 8: 257-266.

Bennet, M.R., Davey, E.F. and Uebel, K.E. (1980) The growth of segmental nerves from the brachial myotomes into the proximal muscles of the chick forelimb during development. J. Comp. Neurol. 189: 335-357.

Bentley, D. and Keshishian, H. (1982) Pathfinding by peripheral pioneer neurons in the grasshopper. Science 218: 1082-1088.

Bentley, D. and Toroian-Raymond, A. (1986) Disoriented pathfinding by pioneer neuron growth cones deprived of filopodia by cytochalasin treatment. Nature 323: 712-715.

Bergen, L.G. and Borisy, G.G. (1983) Tubulin-colchicine complex inhibits microtubule elongation at both plus and minus ends. J. Biol. Chem. 258: 4190-4194.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V., and Rosenthal, A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates *trk* and *trk*B. Neuron 7: 857-866.

Bixby, J.L. (1989) Protein kinase C is involved in laminin stimulation of neurite outgrowth. Neuron 3: 287-297.

Black, I.B. (1978) Regulation of autonomic development. Ann. Rev. Neurosci. 1: 183-214.

Black, M.M. and Lasek, R.J. (1980) Slow components of axonal transport: two cytoskeletal networks. J. Cell Biol. 86: 616-623.

Bozyczko, D. and Horwitz, A.F. (1986) The participation of a putative cell surface receptor for laminin and fibronectin in peripheral neurite extension. J. Neurosci. 6: 1241-1251.

Bray, D. (1970) Surface movements during the growth of single explanted neurons. Proc. Natl. Acad. Sci. USA 65: 905-910.

Bray, D. (1979) Mechanical tension produced by nerve cells in tissue culture. J. Cell Sci. 37: 391-410.

Bray, D. (1982) Filopodial contraction and growth cone guidance. In R. Bellairs (Ed.), Cell behaviour, pp. 229-317. CUP, Cambridge, UK.

Bray, D. (1984) Axonal growth in response to experimentally applied tension. Dev. Biol. 102: 379-389.

Bray, D. (1987) Growth cones: do they pull or are they pushed? Trends Neurosci. 10: 431-434.

Bray, D. (1991) Cytoskeletal basis of nerve axon growth. In P.C. Letourneau (Ed.), The nerve growth cone, pp. 7-17. Raven Press, New York.

Bray, D. and Chapman, K. (1985) Analysis of microspike movements on the neuronal growth cone. J. Neurosci. 5: 3204-3212.

Bray, D. and Hollenbeck, P.J. (1988) Growth cone motility and guidance. Ann. Rev. Cell Biol. 4: 43-61.

Bray, D., Money, N.P., Franklin, M.H. and Bamburg, J.R. (1991) Responses of growth cones to changes in osmolality of the surrounding medium. J. Cell Sci. 98: 507-515.

Bronner-Fraser, M. and Fraser, S.E. (1988) Cell lineage analysis reveals multipotency of some avian neural crest cells. Nature 335: 161-164.

Bronner-Fraser, M. and Lallier, T. (1988) A monoclonal antibody against a laminin-heparan sulphate proteoglycan complex perturbs cranial neural crest cell migration *in vivo*. J. Cell Biol. 106: 1321-1329.

Bunge, M.B., and Bray, D. (1981) Serial analysis of microtubules of cultured rat sensory neurons. J. Neurocytol. 10: 589-605.

Burmeister, D.W., Rivas, R.J. and Goldberg, D.J. (1991) Substrate bound factors stimulate engorgement of growth cone lamellipodia during neurite elongation. Cell Motil. Cytoskel. 19: 255-268.

Burstein, D.E. and Greene, L.A. (1978) Evidence for both RNA-synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. Proc. Natl. Acad. Sci. USA 75: 6059-6063.

Cambray-Deakin, M.A. and Burgoyne, R.D. (1987) Posttranslational modifications of α-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. J. Cell Biol. 104: 1569-1574.

Campenot, R.B. (1982) Development of sympathetic neurons in compartmentalized cultures. I: local control of neurite growth by nerve growth factor. Dev. Biol. 93: 1-12.

Carbonetto, S., Gruver, M.M. and Turner, D.C. (1983) Nerve fibre growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. J. Neurosci. 3: 2324-2335.

Caroni, P. and Schwab, M.E. (1988)a Antibody against myelin-associated inhibitor of neurite growth neutralizes non-permissive substrate properties of white matter. Neuron 1: 85-96.

Caroni, P. and Schwab, M.E. (1988)b Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J. Cell Biol. 106: 1281-1288.

Carr, V.M., and Simpson, S.B. (1978)a Proliferative and degenerative events in the early development of chick dorsal root ganglia. I: Normal development. J. Comp. Neur.182: 727-740.

Carr, V.M., and Simpson, S.B. (1978)b Proliferative and degenerative events in the early development of chick dorsal root ganglia. II: Responses to altered peripheral fields. J. Comp. Neur. 182. 741-756.

Chang, S., Rathjen, F.G. and Raper, J.A. (1987) Extension of neurites on axons is impaired by antibodies against specific neural cell surface glycoproteins. J. Cell Biol. 104: 355-362.

Charlwood, K.A., Lamont, D.M. and Banks, B.E.C. (1972) Apparent orienting effects produced by nerve growth factor. In E. Zairnius (Ed) NGF and its antiserum, pp. 102-107. Athlone, New York.

Cheng, T.P.O. and Reese, T.S. (1987) Recycling of plasmalemma in chick tectal growth cones. J. Neurosci. 7: 1752-1759.

Clark, P., Connolly, P., Curtis, A.S.G., Dow, J.A.T. and Wilkinson, C.D.W. (1990) Topographical control of cell behaviour. II: Multiple grooved substrata. Dev. 108: 635-644.

Clark, P., Connolly, P., Curtis, A.S.G., Dow, J.A.T. and Wilkinson, C.D.W. (1991) Cell guidance by ultrafine topography *in vitro*. J. Cell Sci. 99: 73-77.

Cohen, S., Levi-Montalcini, R. and Hamburger, V. (1954) A nerve growth-stimulating factor isolated from sarcomas 37 and 180. Proc. Natl. Acad. Sci. USA 40: 1014-1018.

Collins, F. and Garrett, J.E. (1980) Elongating nerve fibres are guided by a pathway of material released from embryonic non-neuronal cells. Proc. Natl. Acad. Sci. USA 77: 6226-6228.

Costello, B., Meymandi, A. and Freeman, J.A. (1990) Factors influencing GAP-43 gene expression in PC12 pheochromocytoma cells. J. Neurosci. 10: 1398-1406.

Coughlin, M.D. and Collins, M.B. (1985) Nerve growth factor dependent development of embryonic mouse sympathetic neurons in dissociated cell culture. Dev. Biol. 110: 392-401.

Curtis, A.S.G. (1967) The cell surface: its molecular role in morphogenesis. Logos, Academic, London.

Curtis, A.S.G. (1973) Cell adhesion. Prog. Biophy. Mol. Biol. 27: 317-386.

Curtis, A.S.G. and Forrester, J.V. (1984) The competitive effects of serum proteins on cell adhesion. J. Cell Sci. 71: 17-35.

Curtis, A.S.G., Forrester, J.V. and Clark, P.C. (1986) Substrate hydroxylation and cell adhesion. J. Cell Sci. 86: 9-24.

Curtis, A.S.G., Forrester, J.V., McInnes, C. and Lawrie, F. (1983) Adhesion of cells to polystyrene surfaces. J. Cell Biol. 97: 1500-1506.

D'Amico-Martel, A. and Noden, D.M. (1983) Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am. J. Anat. 166: 445-168.

Daniels, M. (1972) Colchicine inhibition of nerve fibre formation *in vitro*. J. Cell Biol. 53: 164-176.

Davies, A.M. (1987) Molecular and cellular aspects of patterning sensory neuron connections in the vertebrate nervous system. Dev.101: 185-208.

Davies, A.M. (1988) Role of neurotrophic factors in development. Trends Genet. 4: 139-143.

Davies, A.M. (1989)a Intrinsic differences in the growth rate of early nerve fibres related to target distance. Nature 357: 553-555.

Davies, A.M. (1989)b Neurotrophic factor bioassay using dissociated neurons. In R.A. Rush (Ed), Nerve growth factors, pp.95-109. Wiley, New York.

Davies, A.M. and Lumsden, A.S.G. (1984) Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. J.Comp. Neurol. 223: 124-137.

Davies, A.M. and Lumsden, A.S.G. (1990) Ontogeny of the somatosensory system: origins and early development of primary sensory neurons. Ann. Rev. Neurosci. 13: 61-73.

Davies, A.M., Thoenen, H. and Barde, Y.-A. (1986) Different factors from the central nervous system and periphery regulate the survival of sensory neurons. Nature 319: 497-499.

Davies, A.M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987) Timing and site of nerve growth factor synthesis in developming skin in relation to innervation and expression of the receptor. Nature 326: 353-358.

Davies, J.A., Cook. G.M.W., Stern, C.D. and Keynes, R.J. (1990) Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. Neuron 2: 1-20.

Davies, J.T. and Rideal, E.K. (1961) Interfacial phenomena. Academic Press, New York.

Davis, B.M., Frank, E., Johnson, F.A. and Scott, S.A. (1989) Development of central projections of lumbosacral sensory neurons in the chick. J. Comp. Neurol. 279: 556-566.

Derjaguin, B.V. and Landau, L.D. (1941) Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Acta. Physicochemica. URSS 14: 633-662.

Dohrmann, U., Edgar, D., Sendtner, M. and Thoenen, H. (1986) Muscle derived factors that support survival and promote fibre outgrowth from embryonic chick spinal motor neurons in culture. Dev. Biol. 118: 209-221.

Dunn, G.A. and Ebendal, T. (1978) Contact guidance on oriented collagen gels. Exp. Cell Res. 111: 465-479.

Ebendal, T. (1976) The relative roles of contact inhibition and contact guidance in orientation of axons extending on aligned collagen fibrils *in vitro*. Exp. Cell Res. 98: 159-169.

Ebendal, T. (1977) Extracellular matrix fibrils and cell contacts in the chick embryo: possible roles in orientation of cell migration and axon extension. Cell Tis. Res. 175: 439-458.

Ebendal, T. (1989) Use of collagen gels to bioassay nerve growth factor activity. In R.A. Rush (Ed), Nerve growth factors, pp. 81-93. Wiley, New York.

Ebendal, T. (1992) Function and evolution in the NGF family and its receptors. J. Neurosci. Res. 32: 461-470.

Ebendal, T. and Jacobson, C.-O. (1977) Tissue explants affecting extension and orientation of axons in cultured chick embryo ganglia. Exp. Cell Res. 105: 379-387.

Edelman, G.M. (1984) Modulation of cell adhesion during induction, histogenesis, and perinatal development of the nervous system. Ann. Rev. Neurosci. 7: 339-378.

Edelman, G.M. (1986) Cell adhesion molecules in the regulation of animal form and tissue pattern. Ann. Rev. Cell Biol. 2: 81-116.

Edgar, D., Timpl, R. and Thoenen, H. (1984) The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. 3: 1463-1468.

Edgar, D., Timpl, R. and Thoenen, H. (1988) Structural requirements for the stimulation of neurite outgrowth by two variants of laminin and their inhibition by antibodies. J. Cell Biol. 106: 1299-1306.

Ernsberger, U. and Rohrer, H. (1988) Neuronal precursor cells in chick dorsal root ganglia: differentiation and survival *in vitro*. Dev. Biol. 126: 420-432.

Feldman, E.L., Axelrod, D., Schwartz, M., Heacock, A.M. and Agranoff, B.W. (1981) Studies on the localization of newly added membranes in growing neurites. J. Neurobiol. 12: 591-598.

Fischard, A., Verna, J.-M., Olivares, J. and Saxod, R. (1991) Involvement of a chondroitin sulphate proteoglycan in the avoidance of chick epidermis by dorsal root ganglia fibres: a study using β-D-Xyloside. Dev. Biol. 148: 1-9.

Forscher, P. and Smith, S.J. (1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. 107: 1505-1516.

Frost, D. and Westerfield, M. (1986) Axon outgrowth of embryonic zebrafish neurons is promoted by laminin and inhibited by fibronectin. Soc. Neurosci. Abstr. 12: 1114.

Giaever, I. and Keese, C.R. (1983) Behaviour of cells at fluid interfaces. Proc. Natl. Acad. Sci. USA 80: 219-222.

Godfrey, E.W., Schrier, B.K. and Nelson, P.G. (1980) Source and target cell specificities of a conditioned medium factor that increases choline acetlytransferase activity in cultured spinal cord cells. Dev. Biol. 77: 403-418.

Goldberg, D.J. and Burmeister D.W. (1986) Stages in axon formation: observations of growth of *Aplysia* axons in culture using video-enhanced contrast-differential interference contrast microscopy. J. Cell Biol. 103: 1921-1931.

Goldberg, D.J. and Burmeister D.W. (1988) Growth cone movement. Trends Neurosci. 11: 257-258.

Goldberg, D.J. and Burmeister D.W. (1989) Looking into growth cones. Trends Neurosci. 12: 503-506.

Gordon-Weeks, P.R. (1989) Growth at the growth cone. Trends Neurosci. 12: 238-240.

Graham, D.E. and Phillips, M.C. (1979)a Proteins at liquid interfaces. I: Kinetics of adsorption and surface denaturation. J. Coll. Inter. Sci. 70: 403-414.

Graham, D.E. and Phillips, M.C. (1979)b Proteins at liquid interfaces. III: Molecular structures of adsorbed films. J. Coll. Inter. Sci. 70: 427-439.

Griffin, J.W., Price, D.L., Drachman, D.B. and Morris, J. (1981) Incorporation of axonally transported glycoproteins into the axolemma during nerve regeneration. J. Cell. Biol. 88: 205-214.

Grinnell, F.A. (1978) Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. 58: 65-144.

Grumet, M. and Edelman, G.M. (1984) Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by specific cell adhesion molecules. J. Cell Biol. 98: 1746-1756.

Gundersen, R.W. and Barrett, J.N. (1979) Neuronal chemotaxis: chick dorsal root axons turn toward high concentrations of nerve growth factor. Science 206: 1079-1080.

Gundersen, R.W. and Barrett, J.N. (1980) Characterization of the turning response of dorsal root neurites toward nerve growth factor. J. Cell. Biol. 87: 546-554.

Gundersen, R.W. (1987) Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro*. Dev. Biol. 121: 423-431.

Gundersen, R.W. (1988) Interference reflection microscopic study of dorsal root growth cones on different substrates: assessment of growth cone-substrate contacts. J. Neurosci. Res. 21: 298-306.

Gurney, M.E., Heinrich, S.P., Lee, M.R. and Yin, H. (1986) Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and sensory neurons. Science 234: 566-574.

Hamburger, V. (1934) The effects of wing bud extirpation on the development of the central nervous system in chick embryos. J. Exp. Zool. 68:449-494.

Hamburger, V. (1939) Motor and sensory hyperplasia following limb-bud transplantations in chick embryos. Physiol. Zool. 12: 268-284.

Hamburger, V. and Hamilton, H. (1951) A series of normal stages in the development of the chick embryo. J. Morphol. 88: 49-92.

Hamburger, V. and Levi-Montalcini, R. (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111: 457-501.

Hamburger, V. and Yip, J.W. (1984) Reduction of experimentally induced neuronal death in spinal ganglia of the chick embryo by nerve growth factor. J. Neurosci. 4: 767-774.

Hamburger, V., Brunso-Bechtold, J.K. and Yip, J.W. (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J. Neurosci. 1: 60-71.

Hammarback, J.A., Palm, S.L., Furcht, L.T. and Letourneau, P.C. (1985) Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. J. Neurosci. Res. 13: 213-220.

Hammarback, J.A., McCarthy, J.B., Palm, S.L., Furcht, L.T. and Letourneau, P.C. (1988) Growth cone guidance by substrate-bound pathways is correllated with neuron-to-pathway adhesivity. Dev. Biol. 126: 29-39.

Hantaz-Ambroise, D., Vigny, M. and Koenig, J. (1987) Heparan sulphate proteoglycan and laminin mediate two different types of neurite outgrowth. J. Neurosci. 7: 2293-2304.

Harris, W.A., Holt, C.E. and Bonhoeffer, F. (1987) Retinal axons with and without their somata growing to and arborizing in the tectum of *Xenopus* embryos: a time-lapse video study of single fibres *in vivo*. Dev. 101: 123-133.

Harrison, R.G. (1907) Observations on the living developing nerve fibre. Anat. Rec. 1: 116-118.

Harrison, R.G. (1910) The outgrowth of the nerve fibre as a mode of protoplasmic movement. J. Exp. Zool. 9: 787-846.

Hartwig, J.H. and Stossel, T.P. (1979) Cytochalasin B and the structure of actin gels. J. Mol. Biol. 134: 539-554.

Hatta, K.S., Tagaki, H., Fujisa, W.A. and Takeichi, M. (1987) Spatial and temporal expression pattern of N-cadherin adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev. Biol. 120: 215-227.

Hay, E.D. (1981) Extracellular matrix. J. Cell Biol. 91: S205-S223.

Heard, D., Seaman, G.V.F. and Simon-Reuss, I. (1961) Electrophoretic mobility of cultured mesodermal tissue cells. Nature 190: 1009.

Heidemann, S.R., Landers, J.M. and Hamborg, M.A. (1981) Polarity orientation of axonal microtubules. J. Cell Biol. 91: 661-665.

Heidemann, S.R., Lamoureux, P. and Buxbaum, R.E. (1990) Growth cone behaviour and production of traction force. J. Cell Biol. 111: 1949-1957.

Hendry, I.A., Stoeckel, K., Thoenen, H. and Iversen, L.L. (1974) The retrograde axonal transport of nerve growth factor. Brain Res. 68: 103-121.

Heuer, J.G., Fatemie-Nainie, S., Wheeler, E.F. and Bothwell, M. (1990) Structure and developmental expression of the chicken NGF receptor. Dev. Biol. 137: 287-304.

Hinkle, L., McCaig, C.D. and Robinson, K.R. (1981) The direction of growth of differentiating neurons and myoblasts from frog embryos in an applied electric field. J. Physiol. 314: 121-135.

Hohn, A., Leibrock, J., Bailey, K. and Barde, Y.-A. (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 344: 339-341.

Hollyday, M. (1983) Development of motor innervation of chick limbs. In J.F. Fallon (Ed), Progress in clinical and biological research: limb developmant and regeneration, Vol.110A, pp. 183-193. Liss, New York.

Honig, M.G. (1982) The development of sensory projection patterns in embryonic chick hind limb. J. Physiol. 330: 175-202.

Honig, M.G., Lance-Jones, C. and Landmesser, L. (1986) The development of sensory projection patterns in embryonic chick limb under experimental conditions. Dev. Biol. 118: 532-548.

Hughes, A.F. (1953) The growth of embryonic neurites. A study on cultures of chick neural tissue. J. Anat. 87: 150-162.

Jacobson, M. (1978) Developmental neurobiology, pp. 299-302. Plenum, New York.

Johnson, J.E., Barde, Y.-A., Schwab, M.E. and Thoenen, M. (1986) Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. J. Neurosci. 6: 3031-3038.

Kalchiem, C., Barde, Y.-A., Thoenen, H. and Le Douarin, N.M. (1987) *In vivo* effects of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells. EMBO J. 6: 2871-2873.

Kater, S.B., Mattson, M.P., Cohan, C. and Conner, J. (1988) Calcium regulation of the neuronal growth cone. Trends Neurosci. 11: 315-321.

Kirschner, M. and Mitchison, T. (1986) Beyond self-assembly: from microtubules to morphogenesis. Cell 45: 329-342.

Klebe, R.J., Bentley K.L. and Hanson, D.P. 1987. Fibronectin-mediated attachment of mammalian cells to polymeric substrata. In J.L. Brash (Ed), Proteins at interfaces: physicochemical and biochemical studies. ACS symposium series 343, American Chemical Society, Washington.

Lackie, J.M. (1986) Cell movement and cell behaviour. Allen & Unwin, London.

Lamoureux, P., Buxbaum, R.E. and Heidemann, S.R. (1989) Direct evidence that growth cones pull. Nature 340: 159-162.

Lamoureux, P., Steel, V.L., Regal, C., Adgate, L., Buxbaum, R.E. and Heidemann, S.R. (1990) Extracellular matrix allows PC12 neurite elongation in the absence of microtubules. J. Cell Biol. 110: 71-79.

Landmesser, L. (1984) The development of specific motor pathways in the chick embryo. Trends. Neurosci. 7: 336-339.

Landmesser, L. (1987) Peripheral guidance cues and the formation of specific motor projections in the chick. In S.S. Easter (Ed), From message to mind: directions in neurobiology, pp. 121-133. Sinauer, Sunderland MA.

Landmesser, L. (1991) Growth cone guidance in the avian limb: a search for cellular and molecular mechanisms. In P.C. Letourneau (Ed), The nerve growth cone, pp. 373-386. Raven Press, New York.

Landmesser, L. and Honig, M.G. (1986) Altered sensory projections in the chick hind limb following the early removal of motor neurons. Dev. Biol. 118: 511-531.

Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. Nature 283: 249-256.

Le Douarin, N.M. (1982) The neural crest. CUP, Cambridge, UK.

Letourneau, P.C. (1975)a Possible roles for cell to substratum adhesion in neuronal morphogenesis. Dev. Biol. 44: 77-91.

Letourneau, P.C. (1975)b Cell-to-substratum adhesion and guidance of axonal elongation. Dev. Biol. 44: 92-101.

Letourneau, P.C. (1978)a Chemotactic response of nerve fibre elongation to nerve growth factor. Dev. Biol. 66: 183-196.

Letourneau, P.C. (1978)b Cell-substratum adhesion of neurite growth cones and its role in neurite elongation. Exp. Cell Res. 124: 127-138.

Letourneau, P.C. (1981) Immunocytochemical evidence for colocalization in neurite growth cones of actin and myosin and their relationship to cell-substratum adhesions. Dev. Biol. 85: 113-122.

Letourneau, P.C. and Shattuck, T.A. (1989) Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. Dev. 105: 505-519.

Letourneau, P.C., Shattuck, T.A. and Ressler, A.H. (1987) "Pull" and "push" in neurite elongation: observations on the effects of different concentrations of cytochalasin B and taxol. Cell Motil. Cytoskel. 8: 193-209.

Levi-Montalcini, R. (1964) Growth control of nerve cells by a protein factor and its antiserum. Science 143: 105-110.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. Science 237: 1154-1162.

Levi-Montalcini, R. and Angeletti, P.U. (1963) Essential role of the nerve growth factor in the survival and maintainance of dissociated sensory and sympathetic nerve cells *in vitro*. Dev. Biol. 7: 653-659.

Levi-Montalcini, R. and Booker, B. (1960) Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve-growth promoting factor. Proc. Natl. Acad. Sci. USA 42: 384-391.

Levi-Montalcini, R. and Levi, G. (1943) Recherches quantitatives sur la marche du processus de differenciation des neurones dans les ganglions spinaux de l'embryon de poulet. Arch. Biol. (Liege) 54: 183-206.

Levi-Montalcini, R., Meyer, H. and Hamburger, V. (1954) *In vitro* experiments on the effects of mouse Sarcoma 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. 14: 49-57.

Lewis, J., Chevalier, A., Kieny, M. and Wolpert, L. (1981) Muscle nerve branches do not develop in chick wings devoid of muscle. J. Embryol. Exp. Morph. 64: 211-232.

Lindsay, R.M., Thoenen, H. and Barde, Y.-A. (1985) Placode and neural crest-derived neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Dev. Biol. 112: 319-328.

Lockerbie, R.O. (1987) The neuronal growth cone: a review of its locomotory, navigational and target recognition capabilities. Neuroscience 20: 719-729.

Luduena, M.A. (1973) The growth of spinal ganglion neurons in serum-free medium. Dev. Biol. 33: 470-476.

Lumsden, A.G.S. and Davies, A.M. (1983) Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. Nature 306: 786-788.

Lumsden, A.G.S. and Davies, A.M. (1986) Chemotropic effect of specific target epithelium in the developing mammalian nervous system. Nature 323: 538-539.

MacLean-Fletcher, A. and Pollard, T.D. (1980) Mechanism of action of cytochalasin B on actin. Cell 20: 329-341.

MacRitchie, F. 1987. Consequences of protein adsorption at fluid interfaces. In J.L. Brash (Ed), Proteins at interfaces: physicochemical and biochemical studies. ACS symposium series 343, American Chemical Society, Washington.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip., N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247: 1446-1451.

Manthorpe, M., Engvall, E. Ruoslahti, E., Longo, F.M., Davis, G.E. and Varon, S. (1983) Laminin promotes neurite regeneration from cultured peripheral and central neurons. J. Cell Biol. 97: 1882-1890.

Margolis, R.L. and Wilson, L. (1977) Addition of colchicine-tubulin complex to microtubule ends: mechanism of substoichiometric colchicine poisoning. Proc. Natl. Acad. Sci. USA 74: 3466-3470.

Maroudas, N.G. (1975) Adhesion and spreading of cells on charged surfaces. J. Theoret. Biol. 49: 417-424.

Maroudas, N.G. (1977) Sulphonated polystyrene as an optimal substratum for the adhesion and spreading of mesenchymal cells in monovalent and divalent saline solutions. J. Cell Physiol. 90: 511-520.

Marsh, L. and Letourneau, P.C. (1984) Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. J. Cell Biol. 99: 2041-2047.

Marshall, K.C. (1980) Microorganisms and interfaces. Bioscience 30: 246-249.

Martin, P., Khan, A. and Lewis, J. (1989) Cutaneous nerves of the embryonic chick wing do not develop in regions denuded of ectoderm. Dev. i06: 335-346.

Matus, A. (1988) Microtubule-associated proteins: their potential role in determining neuronal morphology. Ann. Rev. Neurosci. 11: 29-44.

McCaig, C.D. (1986) Dynamic aspects of amphibian neurite growth and the effects of an applied electric field. J. Physiol. 375: 55-69.

McCaig, C.D. (1989) Nerve growth in the absence of growth cone filopodia and the effects of a small applied electric field. J. Cell Sci. 93: 715-721.

McCaig, C.D. and Dover, P.J. (1989) On the mechanism of oriented myoblast differentiation in an applied electric field. Biol. Bull. mar. biol. Lab., Woods Hole 176: 140-144.

Mehrishi, J.N. (1972) Surface molecular components of human lymphocytes. Int. A. Aller. 42: 69-77.

Menesini-Chen, M.G., Chen, J.S. and Levi-Montalcini, R. (1978) Sympathetic nerve fibre growth in the central nervous system of neonatal rodents upon intracerebral NGF injection. Archs. Ital. Biol. 116: 53-84.

Mitchison, T. and Kirschner, M. (1988) Cytoskeletal dynamics and nerve growth. Neuron 1: 761-722.

Moore, W.J. and Eyring, H. (1938) Theory of the viscosity of unimolecular films. J. Chem. Phys. 6: 391-394.

Morrison, R.S., Kornblum, H.I., Leslie, F.M., and Bradshaw, R.A. (1987) Trophic stimulation of cultured neurons from rat brain by epidermal growth factor. Science 238: 319-328.

Nakai, J. (1956) Dissociated dorsal root ganglia in tissue culture. Amer. J. Anat. 99: 81-130.

Nakai, J. and Kawasaki, Y. (1959) Studies on the mechanism determining the course of nerve fibres in tissue culture. I: The reaction of the growth cone to various obstructions. Z. Zellforschung 51: 108-122.

Narayanan, C.H. and Narayanan, Y. (1978) Determination of the embryonic origin of the mesencephalic nucleus of the trigeminal nerve in birds. J. Embryol. Exp. Morphol. 43: 85-105.

Nurcombe, V. and Bennett, M.R. (1983) The growth of neurites from explants of brachial spinal cord exposed to different components of wing bud mesenchyme. J. Comp. Neurol. 219: 133-142.

Oakley, R.A. and Tosney, K.W. (1991) Peanut-agglutinin and chondroitin-6-sulphate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. Dev. Biol. 147: 187-206.

Okabe, S. and Hirokawa, N. (1990) Turnover of fluorescently labelled tubulin and actin in the axon. Nature 343: 479-482.

Oppenheim, R.W., Prevette, D., Barde, Y.-A. and Kolbeck, R. (1992) Neurotrophins and motoneuron survival during avian development. Soc. Neurosci. Abstr. 387.1

Oster, G. (1988) Biophysics of the leading lamella. Cell Motil. Cytoskel. 10: 164-171.

Oster, G. and Perelson, A.S. (1987) The physics of cell motility. J. Cell Sci. Suppl. 8: 35-54.

Pannese, E. (1972) The histogenesis of the spinal ganglia. Adv. Anat. Embryol. Cell Biol. Vol. 47.

Parson, S.H. (1990) Control of growth and development of neurons in the chick embryo. Ph.D. thesis, University of Edinburgh.

Patel, N.B. and Poo, M.-M. (1982) Orientation of neurite growth by extracellular electric fields. J. Neurosci. 2: 483-496.

Pearce, F.L., Banthorp, D.V., Cook, J.M. and Vernon, C.A. (1973) Adsorption of nerve growth factor onto surfaces: implications for assay in tissue culture. Eur. J. Biochem. 32: 569-575.

Pethica, B.A. (1961) The physical chemistry of cell adhesion. Exp. Cell Res. suppl. 8: 123-140.

Pollack, E.D. and Leibig, V. (1977) Differentiating limb tissue affects neurite growth in spinal cord cultures. Science 197: 899-900.

Pollack, E.D. and Muhlach, W.L. (1980) Stage dependency in eliciting target-dependent enhanced neurite outgrowth from spinal cord explants *in vitro*. Dev. Biol. 86: 259-263.

Pollack, E.D. and Muhlach, W.L. (1982) Target control of neuronal development during formation of the spinal reflex arc. J. Neurosci. Res. 8: 343-355.

Pollack, E.D., Leibig, V. and Muhlach, W.L. (1979) Limb target and nerve growth factor influence on nerve fibre growth from frog tadpole sensory ganglia in tissue culture. J. Cell Biol. 83: 135a.

Pollack, E.D., Leibig, V. and Reed, C.R. (1980) Stage-dependent growth influences on frog tadpole dorsal root ganglion neurites exerted by spinal cord explants *in vitro*. Soc. Neurosci. Abstr. 6:377.

Pollack, E.D., Muhlach, W.L. and Leibig, V. (1981) Neurotropic influence of mesenchymal limb target tissue on spinal cord neurite growth *in vitro*. J. Comp. Neurol. 200: 393-405.

Pollard, T.D. and Cooper, J.A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Ann. Rev. Biochem. 55: 987-1035.

Poo, M.-M. and Robinson, K.R. (1977) Electrophoresis of concanavalin A receptors along embryonic muscle cell membrane. Nature 265: 602-605.

Purves, D. (1988) Body and brain: a trophic theory of neural connections. Harvard University Press, Cambridge, USA.

Ramon y Cajal, S. (1890) A quelle epoque apparaissent les expansions des cellule nerveuses de la moelle epinere du poulet. Anat. Anzerger 5: 609-613.

Ramon y Cajal, S. (1929) Etudes sur la neurogenese de quelque vertebres. Madrid: CSIC.

Rappaport, C. (1971) In, M.L. Hair (Ed), Chemistry of biosurfaces, vol.2, pp.449. Dekker, New York.

Recio-Pinto, E., Rechler, M.M. and Ishii, D.N. (1986) Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. J. Neurosci. 6: 1211-1219.

Rickmann, M., Fawcett, J.W. and Keynes, R.J. (1985) The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J. Embryol. Exp. Morphol. 90: 437-455.

Rodriguez-Tebar, A., Jeffrey, P.L., Thoenen, H. and Barde, Y.-A. (1989) The survival of chick retinal ganglion cells in response to brain-derived neurotrophic factor depends on their embryonic age. Dev. Biol. 136: 296-303.

Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J. and Furcht, L.T. (1983) Neurite extension by peripheral and central nervous system neurons in reponse to substrate-bound fibronectin and laminin. Dev. Biol. 98: 212-220.

Rogers, S.L., Edson, K.J., Letourneau, P.C. and McLoon, S.C. (1986) Distribution of laminin in the developing peripheral nervous system of the chick. Dev. Biol. 113: 429-435.

Roncali, L. (1970) The brachial plexus and the wing nerve pattern during early developmental phases in chicken. Monit. Zool. Ital. 4: 81-98.

Rossino, P., Gavazzi, I. and Timpl, R. (1990) Nerve growth factor induces increased expression of a laminin-binding integrin in rat pheochromocytoma PC12 cells. Exp. Cell Res. 189: 100-108.

Rutishauser, U. and Edelman, G.M. (1980) Effects of fasciculation on the outgrowth of neurites from spinal ganglia in culture. J. Cell Biol. 87: 370-378.

Rutishauser, U. and Jessell, T.M. (1988) Neural cell adhesion molecules. Phys. Rev. 68: 819-857.

St. Amand, G.S. and Tipton, S.R. (1954) The separation of neuroblasts and other cells from grasshopper embryos. Science 119: 93-94.

Salton, S.R., Richter-Landsberg, C., Greene, L.A. and Shelanski, M.L. (1983) The NGF-inducible large external (NILE) glycoprotein: studies of a central and peripheral marker. J. Neurosci. 3: 441-454.

Sanes, J.R. (1989) Extracellular matrix molecules that influence neural development. Ann. Rev. Neurosci. 12: 491-516.

Saxod, R. (1978) Combination of cholinesterase staining of nerves and stereoscopic viewing for three dimensional study of skin innervation of whole mount. J. Invest. Dermatol. 70: 95-97.

Schnell, L. and Schwab, M.E. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343: 269-272.

Schubert, D. and Whitlock, C. (1977) Alteration of cellular adhesion by nerve growth factor. Proc. Natl. Acad. Sci. USA 74: 4055-4058.

Schulze, E.S. and Kirschner, M. (1988) Direct observation of microtubule dynamics in living cells. Nature 334: 356-359.

Schwab, M.E. (1990) Myelin-associated inhibitors of neurite growth and regeneration in the CNS. Trends Neurosci. 13: 452-456.

Schwab, M.E. and Caroni, P. (1988) Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblst spreading *in vitro*. J. Neurosci. 8: 2381-2393.

Scott, S.A. (1982) The development of the segmental pattern of skin sensory innervation in the embryonic chick hind limb. J. Physiol. 330: 203-220.

Scott, S.A. (1986) Skin sensory innervation patterns in embryonic chick hindlimb following dorsal root ganglion reversals. J. Neurobiol. 17: 649-668.

Scott, S.A. (1987) The development of skin sensory innervation patterns. Trends Neurosci. 10: 468-473.

Scott, S.A. (1988) Skin sensory innervation patterns in embryonic chick hindlimbs deprived of motoneurons. Dev. Biol. 126: 362-374.

Shaw, G. and Bray, D. (1978) Movement and extension of isolated growth cones. Exp. Cell Res. 104: 55-62.

Silver, J. and Rutishauser, U. (1984) Guidance of optic axons *in vivo* by a preformed adhesive pathway of neuroepithelial endfeet. Dev. Biol. 106: 485-499.

Silver, J. and Sapiro, J. (1981) Axonal guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors. J. Comp. Neurol. 202: 521-538.

Singer, S.J. and Kupfer, A. (1986) The directed migration of eukaryotic cells. Ann. Rev. Cell Biol. 2: 337-365.

Smalheiser, N.R. (1989) Morphologic plasticity of rapid-onset neurites in NG108-15 cells stimulated by substratum bound laminin. Dev. Brain Res. 45:39-47.

Smith, C.L. (1983) The development and postnatal organization of primary afferent projections to the rat thoracic spinal cord. J. Comp. Neurol. 220: 29-43.

Smith, C.L. and Frank, E. (1988) Specificity of sensory projections to the spinal cord during develoment in bullfrogs. J. Comp. Neur. 269: 96-108.

Smith, S.J. (1988) Neuronal cytomechanics: the actin based motility of growth cones. Science 242: 708-715.

Speidel, C.C. (1933) Studies of living nerve. II: Activities of amoeboid growth cones, sheath cells and myelin segments as revealed by prolonged observation of individual nerve fibres in frog tadpoles. Am. J. Anat. 52: 1-75.

Stern, C.D. and Keynes, R.J. (1987) Interactions between somite cells: the formation and maintenance of segment boundaries in the chick embryo. Development 99: 261-272.

Stoeckel, K., Guroff, G., Schwab, M. and Thoenen, H. (1976) The significance of retrograde axonal transport for the accumulation of systematically administered nerve growth factor (NGF) in the rat superior cervical ganglion. Brain Res. 109: 271-284.

Stoppin, L., Buchs, P.A. and Muller, D. (1991) A simple method for organotypic culture of nervous tissue. J. Neurosci. Meth. 37: 173-182.

Straznicky, C. and Rush, R.A. (1985) Nerve growth factor treatment does not prevent dorsal root ganglion cell death induced by target removal in chick embryos. Anat. Embryol. 171: 357-363.

Sugimoto, Y. (1981) Effects on the adhesion and locomotion of mouse fibroblasts by their interacting with differently charged substrates. Exp. Cell Res. 135: 39-45.

Sugimoto, Y. and Hagiwara. A. (1979) Cell locomotion on differently charged substrates. Exp. Cell Res. 120: 245-252.

Swanson, G. and Lewis, J. (1982) The timetable of innervation and its control in the chick wing bud. J. Embryol. Exp. Morphol. 71: 121-137.

Swanson, G. and Lewis, J. (1986) Sensory nerve roots in chick wing buds deprived of motor innervation. J. Embryol. Exp. Morphol. 95: 37-52.

Takeichi, M. (1987) Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. Trends Genet. 3: 213-217.

Tello, J.F. (1922) Die Entstehung der motorischen und sensiblen Nervenendigungen. I: In dem lokomotorischen system der hoheren Wirbeltiere: Muskulare histogenese. Z. Anat. Entw. Gesch. Organ. 64: 248-440.

Tessier-Lavigne, M., Placzek, M., Lumsden, A.G.S., Dodd, J. and Jessell, T. (1988) Chemotropic guidance of developing axons in the mammalian central nervous system. Nature 336: 775-778.

Thiery, J.-P., Brackenbury, R., Rutishauser, U. and Edelman, G.M. (1977) Adhesion among neural cells of the chick embryo. II: Purification and characterization of a cell adhesion molecule from neural retina. J. Biol. Chem. 252: 6841-6845.

Thoenen, H. (1991) The changing scene of neurotrophic factors. Trends Neurosci. 14: 165-170.

Tilney, L.G. and Inoue S. (1982) Acrosomal reaction of *Thyone* sperm. II: The kinetics and possible mechanism of acrosomal process elongation. J. Cell Biol. 93: 820-827.

Tomaselli, K.J., Neugebauer, K.M., Bixby, J.L., Lilien, J. and Reichardt, L.F. (1988) N-cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. Neuron 1: 33-43.

Torimitsu, K. and Kanawa, A. (1990) Selective growth of sensory nerve fibres on metal oxide pattern in culture. Dev. Brain Res. 51: 128-131.

Tosney, K.W. (1988) Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: partial and complete deletion of the somite. Dev. Biol. 127: 266-286.

Tosney, K.W. and Landmesser, L. (1985) Development of the major pathways for neurite outgrowth in the chick hindlimb. Dev. Biol. 109: 193-214.

Tsukita, S. and Ishikawa, H. (1981) The cytoskeleton in myelinated axons: serial section study. Biomed. Res. 2: 424-437.

Turner, D.C. and Flier, L.A. (1989) Receptor-mediated active adhesion to the substratum is required for neurite outgrowth. Dev. Neurosci. 11: 300-312.

Tuttle, R. and Matthew, W.D. (1991) An *in vitro* bioassay for neurite growth using cryostat sections of nervous tissue as a substratum. J. Neurosci. Meth. 39: 193-202.

Van hoof, C.O., De Graan, P.N., Boonstra, J., Oestreicher, A.B., Schmidt-Michels, M.H. and Gispen, W.H. (1986) Nerve growth factor enhances the level of the protein kinase C substrate B-50 in pheochromocytoma PC12 cells. Biochem. Biophys. Res. Comm. 139: 644-651.

Verna, J.-M. (1985) *In vitro* analysis of interactions between sensory neurons and skin: evidence for selective innervation of dermis and epidermis. J. Embryol. Exp. Morphol. 86: 53-70.

Verna, J.-M. and Saxod, R. (1979) Development of cutaneous innervation in the chick: ultrastructural and quantitative analysis. Arch. Anat. Microsc. Morph. Exp. 68: 1-16.

Verna, J.-M., Fischard, A. and Saxod, R. (1989) Influence of glycosaminoglycans on neurite morphology and outgrowth patterns *in vitro*. Int. J. Dev. Neurosci. 7: 389-399.

Visintini, F. and Levi-Montalcini, R. (1939) Relazione tra differenziazione strutturale e funzionale dei centri e delle vie nervose nell' embrione di pollo. Schwiez. Arch. Neurol. Neurochir. Psychiat. 43: 381-393.

Vogel, K.S. and Davies, A.M. (1991) The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. Neuron 7: 819-830.

Walicke, P.A., Cowan, W.M., Ueno, N., Baird, A. and Guilleman, R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA 83: 3012-3016.

Walicke, P.A. (1989) Novel neurotrophic factors, receptors and oncogenes. Ann. Rev. Neurosci. 12: 103-126.

Westerfield, M. and Eisen, J.S. (1988) Neuromuscular specificity: pathfinding by identified motor growth cones in a vertebrate embryo. Trends Neurosci. 11: 18-22.

Weston, J.A. (1963) A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. Dev. Biol. 6: 279-310.

Wright, E.M., Vogel, K.S. and Davies, A.M. (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9: 139-150.

Wyatt, S., Shooter, E.M. and Davies, A.M. (1990) Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. Neuron 2: 421-427.

Yamada, K.M. (1983) Cell surface interactions with extracellular materials. Ann. Rev. Biochem. 52: 761-799.

Yamada, K.M., Spooner, B.S., Wessells, N.K. (1970) Axon growth: Roles of microfilaments and microtubules. Proc. Natl. Acad. Sci. USA 66: 1206-1212.

Yamada, K.M., Spooner, B.S., Wessells, N.K. (1971) Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol. 49: 614-635.

Yip, H.K. and Johnson, E.M. (1984) Developing dorsal root ganglion neurons require trophic support from their central processes: evidence for a role of retrogradely transported nerve growth factor from the central nervous system to the periphery. Proc. Natl. Acad. Sci. USA 81: 6245-6249.

Yip, J.W. and Yip, Y.P.L. (1990) Changes in fibronectin distribution in the developing peripheral nervous system of the chick. Dev. Brain Res. 51: 11-18.

Zenker, W. and Hohberg, E. (1973) A-α-nerve-fibre: number of neurotubules in the stem fibre and in the terminal branches. J. Neurocytol. 2: 143-148.

Zieve, G.W., Turnbull, D., Mullin, J.M. and McIntosh, J.R. (1980) Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Exp. Cell Res. 126: 397-405.

Landreth, G.E. and Agranoff, B.W. (1979) Explant culture of adult goldfish retina: a model for the study of CNS regeneration. Brain Res. 161: 39-53.

Mitchison, T. (1989) Poleward microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. J. Cell Biol. 109: 637-652.

Okabe, S. and Hirokawa, N. (1992) Differential behaviour of photoactivated microtubules in growing axons of mouse and frog neurons. J. Cell Biol. 117: 105-120.

Reinsch, S.S., Mitchison, T. and Kirschner, M. (1991) Microtubule polymer assembly and transport during axonal elongation. J. Cell Biol. 115: 365-379.

Popov, S., Brown, A. and Poo, M.-M. (1993) Forward plasma membrane flow in growing nerve processes. Science 259: 244-246.